A FUNCTIONAL AND IMMUNOLOGICAL ANALYSIS
OF THE PROLIFERATING CELL NUCLEAR ANTIGEN

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ABSTRACT

Proliferating Cell Nuclear Antigen (PCNA) is a cell cycle regulated protein involved in DNA replication. The cDNA of rat PCNA was subcloned in various expression vectors. A fusion protein of PCNA with β-galactosidase was produced by subcloning rat PCNA cDNA in plasmid pUR288. The PCNA cDNA was also expressed as protein A fusion protein. PCNA in an unfused form was produced by subcloning rat PCNA cDNA in a T7 polymerase based expression system.

These bacterially expressed fusion proteins were used to immunise Balb/c mice which were then used to raise monoclonal antibodies against PCNA. Eleven new monoclonal anti-PCNA antibodies were raised and designated PC1 to PC11. All the eleven antibodies recognised denatured antigen on a western blot. Ten antibodies recognised native antigen in an immunoprecipitation reaction. Antibodies PC4, PC7 and PC9 do not stearically compete with the rest of the anti-PCNA antibodies. Immunofluorescence studies performed with these antibodies showed three distinct patterns. A large group comprising of PC1, PC2, PC3, PC4, PC5, PC6, PC8, PC10 and PC11 showed nuclear staining which showed cell cycle variation. PC7 failed to give any positive reaction on immunofluorescence while PC9 showed only nucleolar staining. Monoclonal antibodies PC2, PC3, PC5, PC8 and PC10 recognised PCNA from Schizosaccharomyces pombe and Spodoptera frugiperda.

One of the antibodies showing strongest reactivity with PCNA in S. pombe, PC10, was used to screen a S. pombe lambda gt11 library. A cDNA of PCNA homologue in S. pombe was isolated. Sequence comparison with other known PCNA cDNA showed that this cDNA was incomplete at its 5'end. This was then used to screen a S. pombe genomic library. A PCNA gene was isolated, sequenced and analysed. This 2.1 kb gene contains a single intron. Gene disruption experiments were carried out in S. pombe. The PCNA gene was found to be essential for the viability of S. pombe.
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ABBREVIATIONS

ARS: Autonomous replicating sequence
ATP: Adenosine 5'-triphosphate
bp: Base pair
BrdU: Bromodeoxyuridine
BSA: Bovine serum albumin
cDNA: Complementary DNA
DAB: 3,3'-Diaminobenzidine
DBP: DNA binding protein
DEPC: Diethyl pyrocarbonate
DHFR: Dihydrofolate reductase
DMSO: Dimethylsulphoxide
DNase: Deoxyribonuclease
dNTP: Deoxynucleotide 5'-triphosphate
DTT: Dithiothreitol
EDTA: Ethylene diamine tetracetic acid
ELISA: Enzyme linked immunosorbent assay
FCS: Foetal calf serum
GTP: Guanosine 5'-triphosphate
HEPES: N-[2-Hydroxyethyl]piperazine-N'-[2-ethanesulphonic acid]
IPTG: Isopropyl β-D-thiogalactoside
kd: Kilodalton
MOPS: 3-[N-Morpholino]propanesulphonic acid
MPF: Maturation promoting factor
NP40: Nonidet P40
CD: Optical density
PBS: Phosphate buffered saline
PCNA: Proliferating cell nuclear antigen
PCR: Polymerase chain reaction
PIPS: Piperazine-N,N'-bis[2-ethanesulfonic acid]
PMSF: Phenylmethylsulphonyl fluorde
pol α: DNA polymerase α
pol δ: DNA polymerase δ
pol ε: DNA polymerase ε
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<tr>
<td>PP2A</td>
<td>Phosphatase type 2A</td>
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<td>RFA</td>
<td>Replication Factor A</td>
</tr>
<tr>
<td>RFC</td>
<td>Replication factor C</td>
</tr>
<tr>
<td>RNase</td>
<td>Ribonuclease</td>
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<tr>
<td>RNasin</td>
<td>RNase inhibitor</td>
</tr>
<tr>
<td>RPA</td>
<td>Replication Protein A</td>
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<tr>
<td>SDS</td>
<td>Sodium dodecyl sulphate</td>
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<td>SDS-PAGE</td>
<td>SDS polyacrylamide gel electrophoresis</td>
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<td>SLE</td>
<td>Systemic lupus erythomatosus</td>
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<td>SSB</td>
<td>Single strand binding protein</td>
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<td>SSC</td>
<td>Standard saline citrate</td>
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<td>SV40</td>
<td>Simian virus 40</td>
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<tr>
<td>TEMED</td>
<td>N,N,N',N'-Tetramethylethylenediamine</td>
</tr>
<tr>
<td>TMB</td>
<td>3,3',5,5'-Tetramethylbenzidine</td>
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<tr>
<td>UV</td>
<td>Ultraviolet</td>
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<tr>
<td>ATF</td>
<td>Activation transcription factor</td>
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<tr>
<td>CREB</td>
<td>Cyclic AMP-responsive element binding protein</td>
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DEDICATED TO MY MOTHER
Proliferating cell nuclear antigen (PCNA) is a nuclear protein that plays an important role in DNA replication. To appreciate its importance it is essential to understand the cell cycle in general and DNA replication in particular. This chapter gives a background of the cell cycle and DNA replication and describes in detail PCNA.

1.1: THE CELL CYCLE

The cell cycle is a set of events which leads to the duplication of cells. It can be divided into two broad stages, the mitotic phase and interphase, although there seems to be no boundary between the start of one phase and the end of the preceding phase. Mitotic phase includes the nuclear division and cytokinesis. This phase includes prophase, prometaphase, metaphase, anaphase and telophase. DNA synthesis occurs in the S phase of the interphase. There is a gap phase, G1, between M phase and S phase and another gap, G2, between S phase and M phase. Biochemical and genetic approaches have been applied to understand the events of the cell cycle. The systems used for the biochemical studies were amphibian and marine eggs (Murray, 1989) whereas the yeasts (Hartwell, 1978, Hayles and Nurse, 1986) were used to understand this process through a genetic approach. Combination of these two approaches gives an overall view of the cell cycle as it occurs in all organisms.

The amphibian and marine oocytes represent a synchronised system which is useful in cell cycle studies (Murray, 1989). The fully grown oocyte is arrested in G2 phase. Secretion of progesterone by the follicles induces the oocyte to undergo meiosis I and it is arrested again in the metaphase of meiosis II. At this stage the unfertilized egg from the ovary is released. Fertilisation of the egg result in the release of the metaphase arrest and the egg then undergoes a series of nearly synchronous divisions without any detectable G1 and G2 phase until after 12 division the divisions become more complex and asynchronous.
The technique of injection into the oocytes and immature eggs led to the identification of various proteins involved in the maturation of the eggs. One of the factors to be identified this way was maturation promoting factor (MPF) (Masui and Markert, 1971, Reynhout and Smith, 1974). The MPF activity fluctuates as the oocyte passes through the embryonic cell cycle. The MPF activity is increased at meiosis I. After meiosis I the level of MPF decreases and again increases and is stably maintained during meiosis II until the metaphase arrest (Reynhoult and Smith, 1974). MPF is found both in meiotic and mitotic cell cycles. In the mitotic cycle MPF activity increases as the cell enters mitosis and decreases as the cell leaves mitosis and again increases during the interphase (Reynhoult and Smith, 1974, Masui and Markert, 1971). MPF has been shown to be a protein kinase that phosphorylates histone H1 (Lohka et al., 1988). It is suggested that the catalytic subunit of MPF is p34^{cdc2} (Gautier et al., 1990), the homologue of the cdc2 gene product in fission yeast. Injection of MPF in protein synthesis arrested embryos induced all the events of mitosis suggesting that protein synthesis is not required for the activation of MPF and that it is the key regulator of mitosis (Maike-Lye et al., 1983).

Cyclins are another set of proteins first identified in sea urchin eggs (Evans et al., 1983). They show variation in their concentration throughout the cell cycle. Their level increases as the cell enters first mitotic division and disappears at the end of mitosis, accumulates during interphase and disappear again at the next mitosis. Based on the sequence of these proteins, they are subdivided into cyclin A and cyclin B and most organisms have both types of cyclins (Booher and Beach, 1988, Hagan et al., 1988, Lehner and O'Farrell, 1989, Minshull et al., 1989, Pines and Hunt, 1987, Swenson et al., 1986). Injection of cyclin A into Xenopus oocytes (Swenson et al., 1986) and cyclin B mRNA into sea urchin eggs (Pines and Hunt, 1987) induced maturation. However, this maturation required protein synthesis thus ruling out the possibility that cyclins are MPF, which causes maturation in the absence of protein synthesis. It was also shown that cyclin synthesis was essential for the activation of MPF in mitosis and meiosis (Murray and Kirschner, 1989b). Cell free extracts made from activated frog eggs were capable of carrying out multiple cell cycles (Hutchison et al., 1988, Murray and Kirschner, 1989a) that showed all the characteristic changes associated with the cell cycle, like nuclear and chromosomal morphology, DNA replication (Murray and Kirschner, 1989a) and oscillations in MPF (Hutchison et al., 1988). When the endogenous
mRNA was destroyed in these extracts the cells were arrested in the interphase. Addition of mRNA for cyclin into this depleted extracts lead to the resumption of the cell cycle (Hutchison et al., 1988). Thus cyclin was necessary and sufficient for the entry into mitosis. Degradation of cyclin was essential for the cells to exit from mitosis (Murray et al., 1989). A mutant cyclin having a 90 amino acid deletion at its N terminus was capable of inducing MPF but was incapable of being degraded. The cells having this type of cyclin were arrested at metaphase (Murray et al., 1989).

A simple model had been proposed to explain all these observations. It is postulated that there is an MPF inactivase present in the cell and its activity remains constant throughout the cell cycle (Murray, 1987). During interphase mRNA translation of cyclin causes the cyclin to reach a threshold level which then activates MPF. The activation of MPF probably causes phosphorylation of some proteins involved in structural changes during mitosis like nuclear envelope breakdown and chromosomal condensation. The increase in concentration of MPF might also trigger the degradation of cyclin. The degradation of cyclin then leads to the inactivation of MPF by the constitutively present inactivase. Phosphatases then remove the phosphates from the substrates and this leads to the return of the interphase state. The loss of MPF results in the decreased degradation of cyclin which then accumulates for the next cycle. Although this model explains most of the observations, no inactivase has yet been detected. If cyclin is required to maintain the activity of MPF then the presence of inactivase is not essential.

Genetic approaches for studying the cell cycle have concentrated on S. pombe (Hayles and Nurse, 1986) and S. cerevisiae (Hartwell, 1978). A large number of cdc mutants have been isolated in which the cell cycle was arrested at several distinct stages. A detailed analysis of these mutants gave rise to a general principle. For the initiation of any step in the cell cycle the preceding step should be complete. Thus DNA synthesis has to be complete for the cells to enter mitosis and proper assembly of the mitotic spindles is essential for the mitosis to be complete (Hartwell, 1978, Hartwell and Weinert, 1989).

Analysis of mutants of budding and fission yeasts have revealed that the cell growth and other events of the cell cycle, e.g., DNA synthesis, centrosome assembly and mitosis are linked (Pringle and Hartwell, 1981). Before starting DNA replication if an alternative developmental fate, like mating and sporulation, is preferred then DNA synthesis is not initiated.
(Bartlett and Nurse, 1990). There is a control point in the G1 called Start at which the cellular environment is assessed. If the proper nutrients are available then the cells traverse the Start point and are then committed to complete the cell cycle. Beside nutrients, mating pheromones also inhibit the entry into Start (Pringle and Hartwell, 1981). Several mutants had been isolated in which the cell cycle was block before Start. In budding yeast, two type of mutants had been identified that arrest before Start point. The first group arrested as non-growing unbudded cells (Matsumoto et al., 1983). The genes from these mutants seem to be involved in the generation of signals that report the nutrient availability. The other type of mutants cells arrested as growing unbudded cells mimicking the effect of the presence of pheromones (Reed, 1980).

The activity of \textit{CDC28} in \textit{S. cerevisiae} also seem to be required for the cell to pass through the Start (Bartlett and Nurse, 1990). \textit{CDC28} is postulated to be similar to MPF in inducing mitosis. In fission yeast the functional equivalent of \textit{CDC28} is \textit{cdc2} which was required both at the Start and at mitosis (Nurse and Bisset, 1981). These two genes can complement the function of each other suggesting their functional equivalence (Beach et al., 1982). The \textit{cdc2} homologue from human can also complement the \textit{cdc2} mutations in fission yeast (Lee and Nurse, 1987). The \textit{cdc2} homologue found in other organisms are designated as p34\textsuperscript{cdc2}. The gene products of these genes show homology to protein kinases (Hindley and Phear, 1984). The level of protein kinase activity immunoprecipitated by the p34\textsuperscript{cdc2} antisera show cell cycle variations. Their activity rather than quantity are essential for the cell to pass Start (Simanis et al., 1987). In fission yeast the gene product of \textit{cdc13} is also required at mitosis. A physical interaction is shown to occur between \textit{cdc2} and \textit{cdc13} (Booher et al., 1989).

There are other genes identified in fission yeast which are not essential but regulate the entry into mitosis. Two genes, \textit{wee1} and \textit{cdc25}, are not essential for the viability of the cell (Russell and Nurse, 1986, Russell and Nurse, 1987b). Varying the ratio of the gene product of these two genes results in the alteration of the size of the cell at which it enters mitosis (Russell and Nurse, 1986). Increasing the ratio of \textit{wee1} to \textit{cdc25} activity leads to the increase in the size required for the cell to enter mitosis whereas decrease in the ratio leads to decrease in the critical size. Another gene \textit{nim1} negatively regulates the activity of \textit{wee1} (Russell and Nurse, 1987a). The gene product of \textit{wee1} and \textit{nim1} show homology with the
known protein kinases and thus phosphorylation seem to play an important part in the regulation of mitosis. The homologues of wee1 and cdc25 have been identified in budding yeast (Russell et al., 1989). Deletion of cdc25 gene and over-production of wee1 leads to the G2 arrest. This suggests that in budding yeast, like in fission yeast, the beginning of mitosis and Start are distinct events.

To account for these observations a model has been proposed for the interphase to mitosis transition (Russell and Nurse, 1987a). For the entry into mitosis the activities of cdc2 and cdc13 are required. These activities are regulated by the cdc25, nim1 and wee1 gene products. It is not known how the cell exits from the mitosis but phosphatases have been implicated at this stage.

A unified view of the cell cycle is based on the fact that homologous genes had been identified in several species. Thus the homologues of cdc2 have been identified in frogs (Dunphy et al., 1988, Gautier et al., 1988), starfish (Arion et al., 1988, Labbe et al., 1988), humans (Draetta and Beach, 1988), and yeast. The other subunit is identified as cyclin (Booher and Beach, 1988). There is ample evidence that suggest that there is a physical interaction between these subunits (Booher et al., 1989). The level of cdc2 remains constant whereas the level of cyclin varies throughout the cell cycle (Draetta and Beach, 1988). The association of the p34cdc2 and cyclin probably undergoes some posttranslational modifications which leads to the MPF activity. The gene product of cdc25 accelerates these changes and wee1 retards them. Activation of MPF leads to degradation of cyclin and thus return to the interphase state (Murray and Kirschner, 1989a). The product of suc1 gene in fission yeast probably helps in the degradation of MPF (Hayles et al., 1986).

A number of key macromolecules had been identified, whose activities are required for the completion of the cell cycle. To get a complete picture each phase of the cycle has to be studied separately. The information obtained from each event could then be combined to give a detailed understanding of the cell cycle. One of the important phases of the cell cycle is the S phase. In the S phase the DNA content in the nucleus is replicated completely. The length of the S phase varies between species and in different cells within a species (Laskey et al., 1989). Chromosome replication involves complete replication of the DNA along with the complex organization of the chromosome which involves nucleosome assembly and chromosome scaffolds.
Cell fusion experiments strongly suggest that the signal for the entry into the S phase are present in the cytoplasm (Rao and Johnson, 1970). Nuclei entered S phase when they were placed in an S phase cytoplasm. Nuclei from one species were induced to replicate when placed in the S phase cytoplasm of another species (Laskey et al., 1989). *Xenopus* eggs when injected into early embryos were induced to replicate in a manner characteristic of the embryos. On the other hand, G2 nuclei fail to replicate in the S phase cytoplasm suggesting that the DNA template is somehow modified after replication such that it is not recognised by the replication machinery (Rao and Johnson, 1970).

DNA replication occurs once during the S phase (Laskey et al., 1989). To elucidate the mechanism of DNA replication two approaches have been followed (Challberg and Kelly, 1989, Stillman, 1989). The first approach involves the use of animal viruses (Stillman, 1989). Progress to understand eukaryotic DNA replication was greatly accelerated by the development of replication competent cell free extract (Li and Kelly, 1985, Stillman and Gluzman, 1985). This cell free replication extract supported the complete replication of simian virus 40 (SV40) DNA and its assembly into chromatin (Stillman and Gluzman, 1985). The replication of SV40 DNA requires only one of the virus encoded proteins, the large tumour (T) antigen, the rest of the enzymes for replication are provided from the host cells (Kelly, 1988). This makes it a useful system for studying in vitro eukaryotic replication of DNA. The second approach utilises cell free extracts of *Xenopus* eggs which is capable of initiation and complete DNA replication of added nuclei (Laskey et al., 1977).

1.2: SV40 DNA REPLICATION

In the S phase DNA replication starts at several points in the eukaryotic genome (Huberman and Riggs, 1986). To understand this process one must have a system having one origin of replication. SV40 DNA is used for this purpose (Kelly, 1988). The genome of SV40 is a circular duplex of approximately 5000 base pairs containing one origin of replication. Except for T antigen its replication makes extensive use of the host replication machinery. SV40 DNA replication takes place in the nucleus of the host cell and the DNA is complexed with histones to form minichromosomes which are indistinguishable from cellular chromatin. The replication starts at the single origin and two replication forks proceed
Bidirectionally. Termination of replication occurs when the two forks meet each other.

In SV40 DNA the replication origin is a 64 base pair segment that contains all the information that is needed for the initiation of its replication. The origin of replication in SV40 DNA contains mainly three distinct regions. The centre region contains four copies of 5-base pair sequence (GAGGC) organised as inverted repeats and is called site I. On one side of this sequence is a 17 base pair AT rich region (site II) which is the site of initial unwinding during replication. On the other side is a 15 base pair imperfect repeat the function of which is unknown (Kelly, 1988).

Plasmids containing SV40 origin of replication can be replicated, in the presence of T antigen, by the host cell replication proteins. Biochemical fractionation of the cell extract followed by the reconstitution of the SV40 DNA replication led to the identification of several cellular proteins involved in replication. A cartoon showing various proteins involved in vitro SV40 DNA replication is shown in Figure 1.1. The replication proteins needed for the complete replication of DNA containing SV40 origin are discussed below.

**T Antigen**

T antigen is expressed early in the infection of SV40 (Stillman, 1989). It induces the quiescent cells to undergo proliferation, causing DNA and ribosomal RNA synthesis. It binds to a number of proteins, e.g., the retinoblastoma gene product (deCaprio et al., 1988), p53 (Lane and Crawford, 1979) and DNA polymerase α/primase complex (Gannon and Lane, 1987, Smale and Tjian, 1986).

T antigen is the only viral protein required for the initiation of replication in SV40 DNA (Stillman, 1989). This 95 kd protein binds to each of the pentamer repeats at the origin (Mastrangelo et al., 1985). In presence of ATP the T antigen probably forms a double hexamer which binds to the pentamer repeats (Dean et al., 1987). This binding results in the local unwinding of site II, the AT rich region. The efficiency of unwinding depends on the precise nucleotide sequence at the AT rich region. Various regions of T antigen are required for the replicative function. Mutation in the zinc finger motif causes inhibition of replication (Loeber et al., 1989). It is speculated that this zinc finger might be involved in the oligomerisation of T antigen.
The activity of SV40 T antigen is probably regulated by phosphorylation. T antigen contains two clusters of phosphorylated serine and threonine residues. One of the clusters is adjacent to the DNA binding domain (Ser 106, 111, 112, 123 and Thr 124) and the other at the C terminus (Ser 639, 676, 677, 679 and Thr 701) (Scheidtmann et al., 1982). Dephosphorylation by alkaline phosphatase causes no effect on its helicase and ATPase activity but increases its ability to bind to the AT rich region and initiate DNA replication (Mohr et al., 1987). Mutation at Ser 123 and Thr 124 causes failure of DNA to replicate, however Thr 124 mutants bind T antigen at the pentamer repeats but were unable to bind to the AT rich region. Ser 677 and 679 mutants showed enhance ability to initiate replication compared to the wild type. Ser 677 mutant fail to bind to site I but binds to site II however Ser 679 mutant bind to site II better than the wild type. Thus the phosphorylation state of the T antigen would generate subsets which would discriminate between two functionally distinct DNA sites (Schirmbeck and Deppert, 1988).

Two important activities associated with T antigen are the helicase (Stahl et al., 1986) and ATPase activities (Giacherio and Hager, 1979). The helicase activity causes the separation of the two strands of DNA which are then stabilised by the binding of SSB (see below). This activity is localised at the C terminus of the T antigen. The helicase moves from 3'-5' direction implicating that it is bound to the leading strand of the DNA (Goetz et al., 1988). The ATPase activity seems to be essential for the DNA replication. Monoclonal antibodies that block the ATPase activity also inhibit DNA replication (Gough et al., 1988, Smale and Tjian, 1986).

These observations strongly suggest that the initial event of replication is the binding of the T antigen to the pentamer motif and the AT rich region. In presence of ATP, T antigen oligomerises and this then causes the early palindrome at the origin to unwind. This unwinding is independent of DNA synthesis. In presence of SSB to stabilise the single stranded DNA and topoisomerase I to relieve the torsional strain an SV40 origin containing plasmid is extensively unwound which when deproteinised is a negatively supercoiled underwound circular molecule (Dean et al., 1990).

**Cellular replication proteins**

Fractionation of human cell extract and subsequent characterisation of the cellular replication factors have led to a detailed understanding of
DNA replication process. Beside the two polymerases, various accessory proteins have been identified.

**Single strand binding protein (SSB)**

Human single strand binding protein (SSB) also called RFA or RPA is a three subunit protein of 70 kd, 34 kd and 11 kd (Fairman *et al.*, 1988, Wobbe *et al.*, 1987, Wold and Kelly, 1988). This protein is implicated in the initiation and elongation steps of replication (Kenny *et al.*, 1990). SSB in the presence of topoisomerase I and T antigen is capable of extensive unwinding of SV40 DNA. This property can be substituted by various other SSB like that of *E. coli*, Ad DBP, herpes simplex virus infected ceil protein, ICP8. However, only human SSB was able to initiate SV40 DNA replication indicating that besides its involvement in unwinding, SSB has some other replicative role. Human SSB is able to stimulate the activity of DNA polymerase α while SSBs from other sources fail to effect polymerase α activity. In presence of ATP the activity of DNA polymerase δ was also stimulated by human SSB in the presence of two other replication proteins, PCNA and RFC (Kenny *et al.*, 1990). This stimulation of polymerase δ in the presence of PCNA and RFC was also observed with other SSBs. Human SSB is shown to increase the specific binding of RFC,PCNA complex to the double stranded primer-template DNA (Tsurimoto and Stillman, 1991b).

Human SSB binds single stranded DNA more efficiently than the double stranded DNA (Kenny *et al.*, 1990). The single stranded DNA binding site on human SSB was located in the 70 kd subunit (Brill and Stillman, 1989). The 34 kd subunit is phosphorylated and this phosphorylation shows cell cycle variations (Din *et al.*, 1990). Monoclonal antibodies to 34 kd were able to inhibit DNA replication suggesting that this subunit is also important in replication. SSB has recently been cloned from yeast (Brill and Stillman, 1989). The subunit structure, chromatographic behaviour, DNA binding activity and cell cycle dependent phosphorylation of the yeast homologue of SSB were similar to human SSB. The 70 kd subunit of yeast SSB stimulates the activity of the yeast strand exchange protein SEP1. However the yeast SSB failed to replace the human SSB in the SV40 replication reaction.

**Topoisomerase I and II**

The importance of topoisomerases in DNA replication came from the studies performed with SV40 DNA *in vivo* (Champoux, 1988, Snapka,
1987) and from the genetic studies on budding and fission yeasts (Thrash et al., 1984, Uemura and Yanagida, 1984). From these studies it was found that topoisomerase I was not essential for the cell viability and for DNA replication. It acts as a swivelase to relieve the torsional strain in the DNA caused by the unwinding of the two strands. Topoisomerase II is an essential enzyme for cell viability. Mutants lacking topoisomerase II have defects in segregation of the daughter DNA molecules. Topoisomerase II can also act as swivelase like topoisomerase I. This has also been demonstrated by using inhibitors for topoisomerase I and II (Yang et al., 1987). Addition of camptothecin, a specific inhibitor of topoisomerase I, does not inhibit DNA replication whereas addition of VM26, an inhibitor of topoisomerase II, causes inhibition of DNA replication. Thus topoisomerases are one of the essential components required for the complete reconstitution of the in vitro SV40 DNA replication (Weinberg et al., 1990, Wobbe et al., 1987).

**DNA polymerases**

A number of DNA polymerases have been purified from various mammalian tissues. They have been classified on the basis of chromatographic behaviour, presence and absence of exonuclease activity, sensitivity to various drugs, template preference and response to associated proteins. They are designated as DNA polymerase α (pol α), DNA polymerase β (pol β), DNA polymerase γ (pol γ), DNA polymerase δ (pol δ) (Fry and Loeb, 1986) and DNA polymerase ε (pol ε) (Nishida et al., 1988). Only pol α and pol δ have been shown to be required for the synthesis of SV40 DNA (Lee et al., 1989, Tsurimoto and Stillman, 1991a, Tsurimoto and Stillman, 1991b). The role of other polymerases are not clear although pol ε and pol β are implicated in DNA repair (Syvaoja, 1990) and because of its location pol γ is believed to be responsible for mitochondrial DNA replication (Syvaoja, 1990).

**DNA polymerase α/primase complex**

Several lines of evidence suggest that pol α plays a primary role in DNA replication (Lehman and Kaguni, 1989): (i) high level of pol α was found in rapidly proliferating tissues, (ii) monoclonal antibodies against pol α when microinjected into cultured mammalian cells inhibited cellular DNA synthesis, (iii) cellular DNA synthesis was inhibited by aphidicolin, a potent inhibitor of pol α, (iv) a mutant mouse cell line containing temperature sensitive pol α was unable to replicate its DNA at the restrictive
temperature, and (v) *in vitro* SV40 DNA replication was inhibited when the extract was depleted of pol α by immunoprecipitation. SV40 DNA replication activity could be restored when reconstituted with purified pol α/primase complex (Murakami *et al.*, 1986b). The SV40 DNA replication activity cannot be restored when pol α/primase complex from a non-permissive host was added suggesting that the host cell specificity was partly determined by the interaction of the host cell pol α/primase complex and the viral T antigen. This was also shown by the fact that the replication of the murine polyomavirus could occur even with non-permissive HeLa extract if beside polyomavirus T antigen, murine pol α/primase complex is added (Murakami *et al.*, 1986a). An *in vitro* association of T antigen and pol α/primase complex had been detected (Smale and Tjian, 1986). This interaction could be blocked by several antibodies against T antigen and also by p53 (Gannon and Lane, 1987).

Pol α/primase complex is composed of four subunits: a large subunit of 180 kd (which is often purified as a group of polypeptides ranging from 185 to 140 kd), and three smaller subunits of 70 kd, 60 kd and 50 kd (Lehman and Kaguni, 1989). The DNA polymerase activity was associated with the large subunit and the primase activity with the 50 and 60 kd subunits (Kaguni *et al.*, 1983). Except for *Drosophila* (Cotterill *et al.*, 1987), the pol α of other species studied thus far lacked 3'-5' exonuclease activity (Lehman and Kaguni, 1989). The cDNA of human polymerase α has been cloned (Wong *et al.*, 1988). This clone showed high degree of amino acid sequence homology with the viral and bacteriophage DNA polymerases. The budding yeast pol I is the counterpart of pol α. This single copy gene was essential for its viability indicating that the neither pol II nor pol III were able to compensate for the function of pol I (Johnson *et al.*, 1985).

The 50-60 kd primase subunit of the mammalian pol α/primase complex was capable of synthesizing 10-15 oligoribonucleotides on a template DNA (Matsumoto *et al.*, 1990). In SV40 origin containing DNA the primer was synthesized *in vitro* at the site where the unwinding of the palindrome sequence by the T antigen takes place. This site was, however, different from the *in vivo* synthesis of the primer (Stillman, 1989). The synthesis of the primer showed a requirement of SV40 T antigen, pol α/primase complex, human SSB and topoisomerase I (Matsumoto *et al.*, 1990). Oligonucleotide synthesis on the template DNA showed a lag corresponding to the lag observed in DNA synthesis. There was an absolute requirement of human SSB. No other SSB, otherwise capable of unwinding
SV40 origin containing DNA, could substitute for the human SSB in the primase dependent oligoribonucleotide synthesis. This showed that this absolute requirement of human SSB in the SV40 replication might be at the primase dependent Okazaki fragment synthesis stage.

The primase activity in the budding yeast was associated with the 48 and 58 kd subunit (Plevani et al., 1985). The genes for the two primase subunits were present in a single copy and were essential for its viability.

Drosophila pol α/primase complex had associated 3'-5' exonuclease activity (Cotterill et al., 1987) in its 185 kd subunit. The exonuclease activity was unmasked only after the removal of 73 kd subunit. Consistent with this observation was the finding that the fidelity of the 185 kd subunit was greater than all its four subunit associated together (Cotterill et al., 1987). This activity was not found in the large subunit of calf thymus pol α/primase complex even after the dissociation of subunits (Lehman and Kaguni, 1989).

Thus pol α/primase complex synthesizes the first oligoribonucleotide strand on the lagging and probably on the leading strand. The DNA is then elongated on this oligoribonucleotide primer by pol α.

DNA polymerase δ

Due to the presence of a 3'-5' exonuclease activity and lack of a primase activity DNA pol δ was readily distinguished from pol α (Lee, 1988). Although not very different in the subunit structure and isoelectric points, these two polymerases had different chromatographic properties and template preferences. DNA pol δ was sensitive to aphidicolin, and was relatively resistant to butylphenyl-dGTP (Lee et al., 1985). One of the characteristics of pol δ was a requirement for PCNA for its activity (Lee et al., 1984). In presence of PCNA the processivity of pol δ was increased about 150 fold when poly(dA).oligo(dT) was used as template/primer. Another polymerase isolated from mammalian tissues showed similar behaviour to that of pol δ but was insensitive to the presence of PCNA. This was later named as pol ε (Nishida et al., 1988).

Pol δ is composed of two polypeptides of about 125 kd and 48 kd (Lee et al., 1984). The fact that these polypeptides were not the proteolytic fragments of other polymerases was ruled out by the peptide map analysis (Wong et al., 1989). Furthermore, these polymerases could be distinguished immunologically (Wong et al., 1989). A monoclonal antibody against pol α (SJK 287-38) was able to neutralise its activity but
did not affect the activity of pol ε. The activity of pol δ was unaffected at lower concentration of this antibody but high concentration did cause some inhibition of its activity. A panel of monoclonal antibodies had recently been raised against pol δ (Lee et al., 1991). One of the monoclonal antibody, A, inhibited the pol δ activity but did not affect the activity of pol α and pol ε thus demonstrating that these polymerases were indeed separate molecules. Monoclonal antibodies had also been raised against pol ε (Lee et al., 1989). One of the monoclonal antibody, #8, was able to neutralise pol ε activity but had no effect on the activity of pol α. However, this antibody was found to be more potent in neutralising the activity of pol δ. Another monoclonal antibody against pol ε, IB5, neutralised the activities of all three polymerases to the same degree suggesting that although these polymerases were separate entities they shared some structural similarities. This is not surprising since mammalian pol α had been shown to have amino acid homology with the remotely related bacterial and viral polymerases (Wong et al., 1988).

Proliferating cell nuclear antigen (PCNA)

PCNA was first identified as an antigen recognised by some autoimmune sera from SLE patients (Miyachi et al., 1978). This protein was later shown to be an accessory factor for pol δ ( Prelich et al., 1987). Bravo and colleagues identified it as a protein that showed cell cycle expression and named it cyclin (Bravo and Graf, 1985). Because of potential confusion with the mitotic cyclins this name should not be used for PCNA.

The role of PCNA in replication came from two line of evidence: the identification of PCNA as an accessory protein for polymerase δ (Lee et al., 1984) and from the in vitro reconstitution of SV40 DNA replication (Prelich et al., 1987). In vitro SV40 DNA replication assay was performed with purified or partially purified proteins. When PCNA was omitted from the in vitro SV40 DNA replication reaction there was no synthesis of the leading strand and length of the lagging strand became shorter (Prelich et al., 1987). This suggested that PCNA was involved in leading strand synthesis and also coordinates the synthesis of leading and lagging strand. PCNA had been shown to associate with RFC to stimulate pol δ (Tsurimoto and Stillman, 1990, Tsurimoto and Stillman, 1991a). An interaction of PCNA, ATP, RFC stimulated the activity of pol δ and this complex inhibited
the activity of pol α suggesting that these proteins might be involved in the polymerase switch (see below) (Tsurimoto and Stillman, 1990).

**Replication factor C (RFC)**

RFC was identified as one of the proteins required for the complete reconstitution of the *in vitro* SV40 DNA replication (Tsurimoto and Stillman, 1989). Omission of this protein from the replication extract inhibited the synthesis of leading and lagging strand also showed aberrant size products suggesting that it was one of the accessory proteins required for the leading strand synthesis and like PCNA, also coordinated leading and lagging strand synthesis. RFC is composed of three subunits with apparent molecular mass of 140, 41 and 37 kd (Tsurimoto and Stillman, 1989). This protein had DNA binding and ATPase activity associated with it (Tsurimoto and Stillman, 1990, Tsurimoto and Stillman, 1991b). The ATPase activity was localised by the UV cross linking of $[^{32}P]$ATP with the 41 kd subunit of RFC. Interaction of PCNA stimulated the ATPase activity of RFC. This activity also showed DNA dependence (Tsurimoto and Stillman, 1991b). The ATPase activity was increased several-fold by either single stranded or double stranded DNA. The preferred substrate seem to be ssDNA annealed to a primer [e.g. poly(dA).oligo(dT)]. The ATPase activity seem to be independent of the primer bound to the template (Tsurimoto and Stillman, 1991b).

DNA binding experiments have shown that the DNA binding activity was located in the 140 kd subunit of RFC. The DNA binding of RFC was dependent on ATP and PCNA. In the absence of ATP, PCNA decreased the amount of RFC-DNA complex formed. In presence of ATP, PCNA increased the complex formation several-fold. Thus ATP hydrolysis seem to regulate the formation of PCNA.RFC.DNA complex.

SV40 DNA replication reaction has been reconstituted with purified proteins (Tsurimoto *et al.*, 1990, Weinberg *et al.*, 1990b). The concentration of these replication proteins seem to be critical for complete reconstitution of SV40 DNA replication reaction (Tsurimoto and Stillman, 1991a, Tsurimoto and Stillman, 1991b). At low concentrations of SSB (less than 12.5 μg/ml) the activity of pol α was stimulated whereas at higher concentration (25 μg/ml) SSB decreased the activity of pol α probably by blocking the non-specific DNA synthesis at the 3' end of the Okazaki fragment. PCNA.RFC complex inhibited the DNA synthesis of leading strand by pol α even at low concentration of SSB. However, the PCNA.RFC
complex along with ATP activated pol δ and synthesis of leading strand occurred. For bimodal DNA synthesis both pol α and pol δ were required (Tsurimoto and Stillman, 1991a, Tsurimoto and Stillman, 1991b).

A model has recently been proposed for the polymerases switching in the leading strand (Tsurimoto and Stillman, 1991a). It was proposed that the pol α in presence of T antigen, SSB and topoisomerase recognises the origin and synthesizes the first Okazaki fragment. RFC seems to be required for the synthesis of Okazaki fragment by pol α. Addition of PCNA to the DNA synthesis reaction containing pol α, SSB, RFC and ATP led to the inhibition of the DNA synthesis by pol α. The binding of this complex at the 3' end however led to the activation of pol δ and the synthesis of DNA was resumed in an ATP dependent manner. Pol α released from the previous strand could start another round of Okazaki fragment synthesis and this process was again repeated.

It has been speculated recently that pol ε is also required for the DNA replication (Syvaoja, 1990). Although no requirement had been found for a third polymerase in the in vitro reconstitution of SV40 DNA replication it is proposed that pol ε might be required for the elongation of the lagging strand or perhaps for the gap filling reaction after the RNA primers have been removed by the host cell RNAse H. Adding the purified pol ε to the in vitro SV40 DNA replication could throw some light on the role and step at which this enzyme is required, if required at all.

**Chromatin assembly factor (CAF-I)**

The replication of eukaryotic DNA requires not only the duplication of DNA but also its correct assembly into the chromatin. Fractionation of the nuclear extract had led to the identification of a protein capable of assembling SV40 DNA into chromatin. This factor is called chromatin assembly factor I (CAF-I) (Smith and Stillman, 1989). This multisubunit protein is composed of 150, 62, 60, 58 and 50 kd polypeptides. When CAF-I was added to the replication reaction mixture containing histones the newly replicated DNA assembled into chromatin like structure as judged by the mobility shift in an agarose gel and resistance to micrococcal nuclease digestion. This assembly process required DNA replication and was specific for the newly replicated DNA. This factor seems to be different from the assembly factors identified in *Xenopus* extract which did not require DNA replication for assembling the DNA into chromatin (Laskey *et al.*, 1977). Sequential addition of histones to the newly replicated DNA occurs for the
chromatin (Smith and Stillman, 1991). Histone 3 and 4 were deposited while the DNA was being replicated and this deposition required CAF-1. Histone H2A and H2B did not require DNA replication for their assembly into the chromatin. Furthermore, it was found that newly synthesized histone H3 and H4 could differentiate between replicated and non-replicated DNA and only replicated DNA was assembled into chromatin (Smith and Stillman, 1991).

Monoclonal antibodies against CAF-I has been raised (Smith and Stillman, 1991). Using these antibodies CAF-1 was found to be associated with the DNA when the histones H3 and H4 were deposited on the DNA and was not present after the deposition of histone H2A and H2B. Based on these results a model has been proposed for the assembly of the chromatin. It has been proposed that after DNA replication CAF-I directed the deposition of histone H3 and histone H4. Further deposition of histone H2A and H2B probably occurs via the exchange of the CAF-I with these histones (Smith and Stillman, 1991). This assembly reaction was performed with CAF-I and an impure nuclear fraction. It was possible that several factors present in this fraction might be required for the assembly.

Other replication associated proteins

The proteins described above when added in the right proportion unwound and elongated two strands of the SV40 DNA. However, they were not able to remove the RNA primers formed by the primase and also the strands were not covalently closed (Weinberg et al., 1990, Tsurimoto et al., 1990). Addition of RNase H and DNA ligase I did not seem to generate completely closed circles (Goulian et al., 1990). Another activity known as circle closing activity (cca) (Goulian et al., 1990) or 5' exonuclease activity (5'exo) (Ishimi et al., 1988) was required for the generation of closed circular DNA. The activities of these proteins were lost during the fractionation procedure. RNase H removed the RNA primer paired with DNA but was unable to break the last DNA-RNA bond. Removal of the last ribonucleotide requires cca or 5' exo and after filling the gaps by a DNA polymerase the resulting DNA could be ligated by DNA ligase I to form closed circular DNA (Goulian et al., 1990).

Thus all these proteins could duplicate the SV40 DNA, although the rate of in vitro replication of SV40 DNA is one tenth of that found in vivo and longer Okazaki fragments are synthesized in vitro.
1.3: CELLULAR DNA REPLICATION

Viral DNA replication had proved to be of immense value in identifying the cellular replication proteins and their role in DNA replication. However, unlike viruses, eukaryotes have multiple origin of replication and all of these origins are activated simultaneously during the S phase. Identifying these sequences have been a great priority in this field. Except for *S. cerevisiae*, no origin of replication has been identified in the eukaryotes.

In *S. cerevisiae* a selective screening procedure was adopted for identifying the origin of replication. A set of sequences known as autonomous replicating sequence (ARS) have been identified (Umek *et al.*, 1989). Plasmids containing these sequences are able to replicate extrachromosomally in *S. cerevisiae* (Brewer and Fangman, 1987). These sequences have a core consensus sequence although flanking region are also important for them to act as an ARS. It was also shown that all the origins of replication are ARS elements but not all ARS elements can act as an origin of replication (Huberman *et al.*, 1988).

Attempts to identify the site for the initiation of DNA replication in higher eukaryotes have not been very successful. Although the origin sequences were not precisely mapped in mammalian cells there does seem to be a precise regulation of replication with respect to DNA sequences (Umek *et al.*, 1989). This was clearly shown in cells containing amplified copies of dihydrofolate reductase (DHFR) genes. Chinese hamster ovary cells grown in the presence of the drug methotrexate had multiple copies of the DHFR genes. One such cell line CHOC400 contained about 1000 copies of DHFR gene arranged as tandem repeats within the chromosome. The high copy number of the gene allowed the identification of a region within the repeats that acts as preferred site for the initiation (Heintz and Hamlin, 1982). These results have been confirmed using several different techniques and also using the cell line having a single copy of the DHFR gene (Burhans *et al.*, 1990).

There seem to be no sequence specificity for the replication origin in unfertilized eggs of *Xenopus* (Harland and Laskey, 1980). Any DNA e.g., plasmid, viral or chromosomal DNA is capable of replicating when microinjected into Xenopus eggs. This might either be due to possible relaxation of sequence specificity of the replication origins during early stages of embryogenesis or due to the presence of short sequences similar to the host cell origins.
The major difference in the SV40 DNA replication apparatus and the cellular DNA replication is the presence of the virus encoded protein, T antigen. T antigen binds to the origin and unwinds the DNA (Stahl et al., 1986). A cellular homologue of T antigen has not yet been identified. It is not known whether the cellular homologue of T antigen would have the origin binding and unwinding properties in the same polypeptide. A number of helicases have been identified in yeast (Sugino et al., 1986, Sung et al., 1989) and mammalian cells (Hubscher and Stalder, 1985, Poll and Benbow, 1988, Seki et al., 1988). It would be interesting to see whether these proteins would be able to substitute for large T antigen in DNA replication assays.

1.4: REGULATION OF DNA REPLICATION

Several lines of evidence suggest that nuclear structure plays an important role in DNA replication (Cook, 1988). The DNA replication sites seem to be immobilised on the nuclear matrix. This was shown by pulse labelling the newly synthesized DNA with either BrdU or biotin-11-dUTP and the incorporated label was immunolocalised by light microscopy. Different section of the same cell showed that replication was restricted to few hundred foci (Mills et al., 1989). These foci were probably formed by clustering of several replication forks and the unreplicated DNA was spooled through these fixed replication foci.

Results obtained with the cell fusion experiments showed that a replicated nuclei could not pass through the S phase again unless it had passed through mitosis (Rao and Johnson, 1970). Eukaryotes in some way could distinguish between replicated and unreplicated DNA. When DNA was injected into *Xenopus* eggs it replicated multiple times when the cell cycle was not arrested on the other hand only once in arrested cells (Harland and Laskey, 1980). Nuclei placed in *Xenopus* egg extract could replicated only once although at different time. The DNA could be made to rereplicate only when the extract was treated with MPF which caused chromatin condensation and nuclear breakdown (Blow and Laskey, 1988). It was also found that nuclear condensation was not essential as permeablisation would also induce DNA to replicate. Based on these results a model had been proposed that a cytoplasmic "licensing" factor was required for the initiation of replication (Blow and Laskey, 1988). This factor lacked a nuclear localisation signal and therefore needed nuclear breakdown to enter and initiate DNA synthesis. Once used in DNA replication it got inactivated.
and the next round of nuclear breakdown was required for more "licensing"
factor to bind DNA and initiate the subsequent round of DNA replication.

Although a considerable amount of information had become available
regarding DNA replication, its regulation still needs to be refined by, for
example, identification of regulatory proteins. Detailed information of DNA
replication and its regulation would probably allow us to understand the
transition from G1 to S phase and also how the cell distinguishes between
replicated and unreplicated DNA.

1.5: PROLIFERATING CELL NUCLEAR ANTIGEN

As described above DNA replication is a complex process requiring
an interplay of several proteins. One such protein required for the
elongation step of DNA replication is PCNA.

1.6: FUNCTIONS OF PCNA

Several lines of evidence suggest that PCNA was involved in DNA
replication. Immunofluorescence analysis using polyclonal or monoclonal
antibodies against PCNA showed that this protein co-localised with the on­
going DNA replication sites within the nucleus (Bravo et al., 1987, Celis
and Celis, 1985). Cells at different stages of the cycle showed different
staining pattern with anti-PCNA antibodies (Celis and Celis, 1985). In G1,
PCNA showed diffuse nuclear localisation which became granular as the cell
progress into the S phase. Some cells showed nucleolar exclusion of PCNA.
However just before the maximum DNA synthesis, the location of PCNA
was dramatically changed into clusters within the nucleus which co­
localised with the nucleolus. As the cell progress into G2 phase the PCNA
staining became granular and diffuse and appeared to fade away in the late
G2 and M phase (Celis and Celis, 1985).

Recently it had been shown that at least two polymerases,
polymerase α and polymerase δ, were involved in in vitro SV40 DNA
replication (Lee and Hurwitz, 1990, Tsurimoto et al., 1990, Weinberg et
al., 1990a). Identification of PCNA as a protein required for the stimulation
of the activity of polymerase δ showed a more direct involvement of PCNA
in DNA replication (Lee et al., 1984, Prelich et al., 1987). DNA polymerase
δ in the absence of PCNA had very little polymerase activity which was
dramatically increased in presence of PCNA. The involvement of PCNA in
DNA replication was also shown by using anti-PCNA antibodies. Addition of
an autoimmune sera, AK, containing antibodies against PCNA in the in vitro
SV40 replication assay inhibited DNA synthesis (Wong et al., 1987). Microinjection of anti-PCNA antibodies into Xenopus oocytes led to the inhibition of plasmid and chromosomal replication (Zuber et al., 1989).

PCNA seems to play a major role in the cell cycle progression. Antisense DNA to the 5' end of the PCNA cDNA blocked the entry of Balb/c 3T3 cells into S phase (Jaskulski et al., 1988a). Similar results were obtained with Chinese Hamster Ovary (CHO) cells (Liu et al., 1989).

Proof of the direct involvement of PCNA in DNA replication came from studies on the reconstitution of SV40 DNA replication with purified or partially purified components (Prelich et al., 1987, Wold et al., 1989). In absence of PCNA only short strands originating from the lagging strand were produced and the synthesis of the leading strand was completely inhibited (Prelich et al., 1987). In presence of PCNA long DNA strands originating from both leading and lagging strand were produced. This suggests that PCNA was involved in the leading strand synthesis and also coordinated the synthesis of the lagging and the leading strands (Prelich et al., 1987).

Using a synthetic template-primer it had been shown that PCNA forms a complex with another replication factor RFC and binding of this complex probably created a site for polymerase δ (Tsurimoto and Stillman, 1991a, Tsurimoto and Stillman, 1991b). RFC also binds and stimulates the activity of polymerase α. It had been shown that binding of PCNA to this complex inhibited this activity and probably removed polymerase α and created the site for polymerase δ. Thus PCNA along with RFC was involved in the leading strand synthesis by polymerase δ and also in the polymerases switching.

Immunofluorescence studies using organic solvents had shown that the PCNA co-localises with the on-going DNA replication sites (Celis and Celis, 1985). However when the cells were fixed with formaldehyde the staining does not seem to show cell cycle variation and quiescent cells seem to have about 30-40% PCNA (Bravo and Macdonald-Bravo, 1987). Thus there seems to be two population of PCNA: one that was involved in DNA replication and not removed during methanol fixation and the free soluble pool which was removed during methanol fixation. The total concentration of PCNA, as determined by western blotting the total cell extract varied only 2-3 fold during the cell cycle. However a greater population of PCNA seem to be associated with the chromosomal DNA during the S phase (Morris and Mathews, 1989). This shows that PCNA
concentration seem to be in far excess of that required for DNA replication. Thus there seem to be another nonreplicative role(s) for PCNA. One of such function may be in DNA repair.

UV irradiation of quiescent cells triggers the appearance of PCNA (Celis and Madsen, 1986, Toschi and Bravo, 1988). When fixed with methanol there was no staining of PCNA in human foreskin fibroblasts. However when these quiescent cells were UV irradiated there was an increase in the PCNA staining. This increase in PCNA levels was independent of DNA synthesis (Toschi and Bravo, 1988). No new PCNA synthesis was required as this effect can also be seen in presence of cyclohexamide. Since PCNA had been shown to be required for stimulation of the activity of polymerase δ (Lee et al., 1984) which had been implicated in DNA repair (Nishida et al., 1988), it can be speculated that PCNA might have a role in DNA repair. The involvement of PCNA directly in DNA repair had not been shown in vitro but proteins involved in DNA replication, e.g., SSB, have recently been shown to be required for DNA repair (Coverly et al., 1991). It would be interesting to see if various cell lines defective in DNA repair, e.g., those derived from Xeroderma pigmentosum patients, have defective PCNA.

1.7: PCNA cDNA AND GENE

PCNA has been identified in various species, e.g., mammals (Almendral et al., 1987, Moriuchi et al., 1986), yeast (Bauer and Burgers, 1990), Xenopus (Leibovici et al., 1990), Drosophila (Yamaguchi et al., 1990) and plants (Suzuka et al., 1989). A protein related to PCNA has also been found in the baculovirus Autographa californica nuclear polyhedrosis virus (O'Reilly et al., 1989). The cDNA from most of species have been cloned and show a high degree of amino acid identity.

Human PCNA cDNA and its gene

A human cDNA for PCNA was cloned from a MOLT-4 cDNA expression library (Almendral et al., 1987). A polyclonal rabbit antibody against MOLT-4 PCNA was used to screen the cDNA library. A cDNA of 1248 bp was isolated and the predicted open reading frame of this cDNA encoded a protein of 261 amino acids. The predicted amino acid sequence of PCNA showed several features of interest. The amino acid sequence from 61-80 showed a helix-turn-helix motif, a putative DNA binding domain. There were 8 leucine repeats at the C terminus of the protein. The
predicted amino acid sequence of PCNA showed some amino acid identity with herpes simplex virus infected cell protein, ICP8, a DNA binding protein (deBruyn Kops and Knipe, 1988). This identity was more at the nucleotide level showing 116 out of 270 nucleotide identity at 5' end. Beside showing amino acid homology PCNA and ICP8 show similar intracellular movement associated with DNA replication. Immunofluorescence using monoclonal antibody against ICP8 show that this protein was re-localised upon the initiation of viral DNA replication (Quinlan et al., 1984). Similar observations were also seen with PCNA (Celis et al., 1988). Infection of monkey kidney epithelial cell line, CV-1 with HSV-1 showed that PCNA and ICP8 were co-localised at the same replication centres (Wilcock and Lane, 1990). This suggests that either these two proteins respond similarly to the same signal or perform similar function during DNA replication.

Using a 300 bp fragment of the human cDNA a PCNA gene was isolated from a human leukocyte genomic library (Traveli et al., 1989). It was shown to be a single copy gene containing 6 exons and 5 introns. The structure of the gene revealed several important features. There was a striking similarity between the introns and between introns and exons. The similarity between intron 1 (733-786) and intron 3 (1616-1667) was 74%; intron 1 (750-787) and intron 4 (3688-3725) 84%; intron 3 (1415-1592) and intron 4 (3611-3786) 67%. All the introns showed some nucleotide similarity with the exons. The 5' flanking region of the PCNA gene was linked to thymidine kinase cDNA to measure the promoter activity of the PCNA gene. Up to 396 bp of 5' flanking region of the PCNA gene was capable of full expression of the reporter gene. Binding site for the transcription factors AP-2 (Mitchell et al., 1987), glucocorticoid responsive element (Chandler et al., 1983) and an octamer motif (Jones et al., 1988) were localised in the 5' flanking region. The octamer motif had been found to be important in the regulation of immunoglobulin and histone H2B (LaBella et al., 1988).

**PCNA cDNA and genes from other species**

Using an oligonucleotide probe corresponding to the first 5 amino acids of rabbit PCNA a cDNA was isolated from rat thymocytes cDNA library (Moriuchi et al., 1986). The predicted open reading frame of this cDNA showed 98.5% amino acid identity to the human protein.
The gene and cDNA for PCNA has also been cloned from Drosophila (Yamaguchi et al., 1990). The predicted open reading frame showed 70% amino acid identity with the predicted amino acid sequence of rat and human PCNA. Using the cDNA of PCNA the level of mRNA was studied at different stage of development of Drosophila. There was high level of mRNA during the first 10 hr of embryonic development. There was a decrease in the PCNA mRNA level in the late embryo through the second instar larvae and this level was further decreased from the third instar larvae to the adults. There were abundant transcripts of PCNA in the unfertilized egg and ovaries suggesting that in the early embryonic stages the transcripts were provided by the maternal gene expression. Drosophila PCNA was also able to complement human PCNA in in vitro SV40 DNA replication (Ng et al., 1990).

Using the cDNA of human PCNA a defolliculated oocyte Xenopus library was screened (Leibovici et al., 1990). A 1033 bp cDNA was isolated. The predicted amino acid sequence showed 89% homology to rat and human PCNA. The PCNA expression during various developmental stages was studied by monitoring the mRNA levels at different stages of development. There was a large stockpile of maternal PCNA mRNA in mature oocyte (3 x 10^7 copies per mature oocyte). After fertilization 90% of the maternal PCNA mRNA was degraded at the gastrula stage and the remaining RNA molecules were distributed among the progeny cells during early cleavage stage. This level was then decreased further and then remained constant till the swimming tadpole stage. Although there was large amount of transcripts in the oocytes translation of PCNA did not start until midoogenesis. After fertilization the PCNA gene product remains constant per embryo till the swimming tadpole stage. Taken together these observation suggest that the level of PCNA could remain high even in the absence of DNA replication.

The gene of PCNA isolated from S. cerevisiae genomic library showed least amino acid homology with all the known PCNAs (Bauer and Burgers, 1990). The yeast PCNA was isolated biochemically and the tryptic fragments of this protein were subjected to peptide sequencing. Degenerate oligonucleotide were then synthesized using the amino acid sequence obtained from this peptide. A genomic S. cerevisiae library was screened with a probe amplified by polymerase chain reaction using these degenerate primers. A 1.1 kb PCNA gene was isolated. The predicted open reading frame showed only 35% amino acid identity with human and rat
PCNA. The monoclonal antibodies raised against mammalian PCNA, 19A2 and 19F4, failed to cross-react with *S. cerevisiae* PCNA further confirming the low amino acid identity. This gene was essential for the viability of *S. cerevisiae*. *S. cerevisiae* PCNA was capable of increasing the processivity of mammalian and yeast polymerase δ (Bauer and Burgers, 1988b). However it failed to complement human PCNA in *in vitro* SV40 DNA replication (Ng *et al.*, 1990). This suggests that beside stimulating polymerase δ human PCNA also performs some other replicative function(s).

Cloning of the PCNA gene and its cDNA had been useful in understanding the structure function relationship. PCNA is a highly conserved protein but this high degree of sequence conservation is not essential for the stimulation of polymerase δ activity. In fact it can be assumed that the residues conserved between human and yeast PCNA are essential for the stimulation of polymerase δ. The conservation of the other residues might be important in the specific protein-protein interactions required for other replicative or non-replicative functions of PCNA.

In mammalian cells the message of PCNA was shown to increase when the cells were in S phase (Celis and Celis, 1985), i.e., at the stage of maximum DNA synthesis. However the synthesis of PCNA mRNA is not dependent on DNA replication (Almendral *et al.*, 1987). Quiescent cells simulated with 10% foetal calf serum in presence of hydroxyurea showed an increase in the level of PCNA mRNA after 10 hr, reaching maximum after 18-20 hrs. Similar pattern was obtained in the absence of hydroxyurea thus showing that DNA synthesis was not required for the synthesis of PCNA mRNA. This can also be seen in *Drosophila* (Yamaguchi *et al.*, 1990) and *Xenopus* (Leibovici *et al.*, 1990) where the oocytes and unfertilized eggs had large amount of PCNA mRNA and maternal stockpiled PCNA was used in early developmental stages when little transcription occurred.

1.8: REGULATION OF PCNA

Systematic analysis of PCNA promoter and its gene revealed that PCNA gene is transcriptionally and post-transcriptionally regulated (Chang *et al.*, 1990). The PCNA gene isolated by Travali *et al* (1989) had 2.8 kb 5' flanking region. This region was capable of driving the synthesis of the reporter thymidine kinase cDNA. Various deletions were made from the 5' end and it was found that a sequence 396 bp upstream of the transcription
start site was capable of driving the synthesis of the reporter thymidine kinase cDNA in TK−ts13 cells suggesting that this sequence was sufficient for the promoter activity of PCNA gene. This sequence had a bidirectional transcriptional activity (Rizzo et al., 1990) and therefore was able to drive the synthesis of the reporter gene in both orientations in vitro. Using sense ribonucleotide as probe within the promoter region some transcription away from the PCNA gene was detected suggesting that this bidirectional promoter activity was also active in vivo. The gene located at the 5' end of the PCNA gene had not been identified. Simultaneous activation of this gene along with the PCNA gene might be essential for the manifestation of its full activity.

The steady state level of PCNA mRNA is growth regulated (Ottavio et al., 1990a). The PCNA gene is responsive to various growth factors, e.g., platelet derived growth factor, fibroblast growth factor and to a less extent to epidermal growth factor (Ottavio et al., 1990a). It was not responsive to insulin. The 5' flanking sequence of the human PCNA gene was analysed to locate the responsive elements present in this region. Various deletions of the promoter region capable of driving the transcription of the reporter gene also responded to the same extent to the growth factors. This suggested that there was no sequence preference for the growth factor response. The combination of growth factors showed the same effect on the transcription as the individual growth factors. Intron 4 of the human PCNA gene was found to be important for the regulation of transcription by the growth factors (Ottavio et al., 1990b). When intron 4 was removed the PCNA gene did respond to the serum but was not turned off when the serum was removed resulting in high steady state level of the PCNA mRNA at Go. This is interesting since, as mentioned before, there is a high degree of nucleotide sequence homology between introns and between introns and exons. Intron 4 shows 60% homology with 300 nucleotides at the 3' end of the human PCNA cDNA (Traveli et al., 1989). It also shows some nucleotide sequence homology with the rest of the introns. Furthermore intron 4 is the longest (1885 bp) of all the introns. It was suggested that there might be a splicing block at the Go in the quiescent cells and this resulted in the hnRNA not being processed into mature mRNA. Removal of intron 4 led to the removal of this block and therefore the PCNA transcripts were also present in Go. Another possibility is the presence of a silencer sequence in intron 4 which is activated during Go (Ottavio et al., 1990b).
The transcripts for PCNA were also induced by interleukin 2 (Shipman et al., 1990) and by E1A protein of ad5 (Jelsma et al., 1989, Morris and Mathews, 1990). The E1A responsive element mapped to 85 nucleotide upstream of the transcription initiation site. Analysis of various motifs which might be involved in this response is an ATF site within that region. An enhancer element between -75 bp and -45 bp of PCNA gene had also been identified. The putative enhancer element had a sequence similar to the SV40 core enhancer and to CREB sequence. A protein had been shown to bind to this sequence (Baserga, 1991).

Recently there was a report that PCNA gene was down-regulated by the wild type p53 (Mercer et al., 1991). Conditional over-expression of wild type p53 in a cell line derived from human glioblastoma multiform tumour blocked the cell cycle progression. One of the consequences of over-expression of wild type p53 was the down-regulation of PCNA mRNA and protein. The PCNA message was lost within 24 hrs after the induction of wild type p53 expression. Since p53 had been shown to possess a potent transactivating function (Fields and Jang, 1990, Raycroft et al., 1990) and over expression of p53 blocked the cell cycle progression, it is proposed that p53 coordinates a set of genes that negatively regulate the key events in the cell cycle progression (Mercer et al., 1991). Therefore down-regulation of PCNA might be one of the direct consequences of over-production of p53 and might be one of the key events in the cell cycle arrest.

1.9: ANTIBODIES AGAINST PCNA

Antibodies against PCNA had proved to be useful in the identification and localisation of PCNA. Three percent of the autoimmune sera from the patients suffering from SLE have antibodies against PCNA (Miyachi, 1978). PCNA was first identified in the nucleus of the cell using these autoimmune sera. Of all the antibodies available against PCNA these antisera proved most useful for functional studies. Since probably they were raised against a native conformation of PCNA some of these sera inhibited in vivo and in vitro DNA replication (Wong et al., 1987, Zuber et al., 1989). One of the sera, AK, had been extensively used in the localisation of PCNA and also in the DNA replication assay (Zuber et al., 1989). The antisera when precleared of all the other contaminating antibodies were able to localise PCNA only in proliferating cells. Cells that have undergone differentiation do not show any PCNA staining (Miyagawa et al., 1989). The AK serum,
recognises the denatured antigen on a western blot and would only immunoprecipitate the full length in vitro translated protein (Huff et al., 1990). The epitope of this antiserum lies between amino acid 99-128 as determined by western blotting the various fragments of PCNA as β-galactosidase-PCNA fusion protein (Huff et al., 1990).

Two monoclonal antibodies, 19A2 (IgM) and 19F4 (IgG1), were produced against rabbit PCNA (Ogata et al., 1987b). These monoclonal antibodies were able to recognise denatured PCNA on western blots but failed to recognise the native protein by immunoprecipitation. Results from the competitive ELISA showed that the epitopes of these antibodies were closely related. Consequently these monoclonal antibodies failed to recognise the antigen captured by any one of them in sandwich ELISA (Ogata et al., 1987a). They were able to recognise PCNA from various species, e.g., rat, human, Xenopus but failed to recognise PCNA from S. cerevisiae (Bauer and Burgers, 1988b). The epitope of 19A2 had been precisely mapped to amino acid 111-125 (Huff et al., 1990). These antibodies do not inhibit in vitro and in vivo chromosomal and plasmid replication (Wong et al., 1987, Zuber et al., 1989).

Recently the production of three monoclonal antibodies, TOB7, TO17 and TO30, against bovine PCNA was reported (Takasaki et al., 1990). The epitope of these monoclonal antibodies were compared with that of 19A2 and 19F4. In competitive ELISA, TO17 and TO30 competed with 19A2 but only partially with 19F4. Antibody TOB7 did not compete with any of the other antibodies. PCNA captured by TOB7 could be recognised by TO17 and TO30 in sandwich ELISA. TOB7 had been used to analyse the proliferative state of PCNA in paraffin embedded formalin fixed section (Murashima et al., 1990). Whether these antibodies would be useful for the functional analysis of PCNA remains to be seen. In this study eleven new monoclonal antibodies were isolated and characterised (Waseem and Lane, 1990).

1.10: IMMUNOHISTOCHEMICAL ANALYSIS OF PCNA

There are several criteria that can be used to assess the proliferative state of the tissue. They are 1: thymidine incorporation followed by autoradiography, 2: BrdU incorporation followed by detection with anti-BrdU antibody, 3: flow cytometry and 4: antibodies against cell cycle markers. For thymidine incorporation and BrdU labelling fresh viable tissues are required whereas fresh or archival tissues could be used for the antibody analysis.
The antibody commonly used for the detection of proliferative cells is Ki-67, an antibody against a nucleolar antigen (Gerdes et al., 1983). This antibody can only be used in the frozen tissues and does not recognise the proliferative cells when the tissue is paraffin embedded and therefore cannot be used on archival materials. Various other antibodies used as proliferation markers include antibodies against DNA polymerase α (Tanaka et al., 1982), ribonucleotide reductase (Engstrom, 1982). Since PCNA shows a cell cycle variation and the protein level seem to peak at the G1/S phase (Celis and Celis, 1985) it had been used as one of the proliferation marker.

Certain tissues, e.g., lymph nodes, and certain cell lines (Landberg et al., 1990) when stained with anti-PCNA antibody showed good correlation with Ki-67 (correlation coefficient =0.9). Depending on the conditions used for fixation of the tissue, most anti-PCNA antibodies, both polyclonal (Landberg et al., 1990) and monoclonal (Ogata et al., 1987a), stained the nuclei of the cells present in the proliferative compartment of the tissue (Garcia et al., 1989). Thus using monoclonal antibody 19A2, PCNA positive cells were found in the germinal centres of methacran fixed tonsil sections (Garcia et al., 1989), within the crypts of small intestine and spermatogonia of testis in which sperms and spermatids showed no PCNA staining. These data suggest that PCNA could be used as an operational marker for cell proliferation. However in certain clinical tissues like breast carcinoma and haemangiopericytomas there seem to be no correlation between the the S phase fraction and PCNA staining (Hall et al., 1990). The reason for this discrepancy is not clear and would require an understanding of the regulation of PCNA gene in these samples.

1.11: AIMS OF THIS STUDY

As described earlier most of our understanding about DNA replication comes from the in vitro studies carried out on SV40 replication. This system is extremely versatile and has provided information about various steps and components involved in the process. To understand the role of PCNA in SV40 DNA replication one needs pure PCNA. Since the biochemical methods of purification give poor yield we decided to express PCNA in E. coli.

The major drawback of SV40 DNA replication system is that it is studied in vitro and therefore does not provide a suitable system to study the linkage of DNA replication with cell growth and division. Yeast and
Drosophila are the system of choice in which a particular gene can be manipulated and its influence on cell cycle studied. A prerequisite for this study was to clone the gene and cDNA for this protein. Since no suitable probes were available we decided to raise monoclonal antibodies against PCNA which will provide us an unlimited source of immunological reagents which could be used in cloning procedure.
Figure 1.1: A diagrammatic representation of the proteins involved in SV40 DNA replication.
CHAPTER 2

MATERIALS AND METHODS

2.1: INTRODUCTION

This chapter deals with the general methods used in the studies described in the later chapters. Specialised methods are described in individual chapters. The protocols described here were taken or modified from "Molecular Cloning, A Laboratory Manual" (Maniatis et al., 1982) and "Antibodies, A Laboratory Manual" (Harlow and Lane, 1988).

2.2: GENERAL PROCEDURES

Autoradiography

Autoradiography was usually performed with Kodak X-Omat AR film at room temperature for $^{35}$S and at $-70^\circ$C for $^{32}$P.

Solutions

Solutions were sterilised by autoclaving, where possible, otherwise by filtration through 0.22μ filters (Nalgene, USA). Protein solutions were stored with 0.02% (w/v) sodium azide.

Dialysis Tubing

Appropriate lengths of dialysis tubing were prepared by boiling in 200 mM sodium bicarbonate and 5 mM EDTA for 5 min. The dialysis tubing was washed with water and boiled again in 200 mM sodium bicarbonate and 5mM EDTA for 5 min. After rinsing with deionised water they were autoclaved and stored at $4^\circ$C.

2.3: DNA CLONING TECHNIQUES

Phenol and phenol/chloroform extraction of DNA

To obtain DNA free from contaminating proteins, phenol and phenol/chloroform extractions were performed. An equal volume of phenol,
containing N-hydroxyquinoline (Sigma Chemical Co.), previously equilibrated with TE, pH 8.0, was added to the solution of DNA and vortexed. After spinning in microfuge at maximum speed for 2 min, the upper aqueous layer was removed and an equal volume of 1:1 mixture of phenol : chloroform was added, mixed and aqueous layer removed. This was then extracted with a mixture of chloroform and isoamyl alcohol (24:1 v/v).

**Ethanol precipitation of DNA**

DNA was precipitated by adding 2.5 volume of ethanol to the DNA solution in 0.3 M sodium acetate, pH 5.5. Depending on the amount of DNA the solution was kept at room temperature or frozen in dry ice for 10 min and centrifuged for 10 min in a microfuge at maximum speed. The DNA pellet was washed with 70% ethanol and dried in a SpeedVac SVC100 (Stratech Scientific, U. K.).

**DNase-free RNase**

A 10 mg/ml stock solution of pancreatic RNase A (Boehringer Mannheim, Germany) was made in 10 mM Tris HCl, pH 7.5 and 15 mM NaCl. This solution was kept in boiling water bath for 10 min and allowed to cool slowly to room temperature. The solution was dispensed in small aliquots and stored at -20°C.

**Preparation of spun Sephadex G-50 column**

Sephadex G-50 was swollen in distilled water, washed several times with TE, pH 8.0 (Table 2.1) and autoclaved. For preparing a spun column Sephadex G-50 gel was packed in a 1 ml syringe and spun at 1600g for 5 min. This procedure was repeated several times until the bed volume remained constant and the volume applied on the column became equal to the volume eluted.

**Plasmid DNA preparation-large scale**

Plasmid DNA was prepared on a large scale by alkaline lysis method essentially according to the method of Birnboim and Doly, (1979). The plasmid preparation was further purified either on a caesium chloride-ethidium bromide gradients or on a Qiagen column (Diagen, Germany).

i) Caesium chloride-ethidium bromide gradient method
A 400 ml culture of bacteria harbouring the required plasmid was grown in L-broth overnight in the presence of an antibiotic at 37°C or at 30°C when plasmid containing a heat inducible promoter was used. The culture was centrifuged at 3000 rpm at 4°C in a Sorvall RC-5B centrifuge using a GSA 600 rotor. The pellet was suspended in 10 ml of 50 mM glucose, 25 mM Tris HCl, pH 8.0, 10 mM EDTA containing 0.10 mg/ml lysozyme (Sigma Chemical Co.) and incubated for 5 min at room temperature. To the lysed cells 20 ml of solution containing 0.2 M NaOH and 1% SDS was added. After incubation for 5 min, 15 ml of 5 M potassium acetate, pH 4.8 was added and mixed thoroughly and allowed to stand for 5 min at room temperature. The precipitate containing denatured chromosomal DNA and cell debris was removed by centrifuging this solution at 5000 rpm in a Sorvall RC-5B centrifuge using an SS-34 rotor for 30 min at 4°C. The supernatant was transferred into a fresh tube and DNA precipitated with 0.6 volumes of isopropanol. After a 15 min incubation at room temperature the solution was centrifuged at 5000 rpm for 30 min. The plasmid DNA pellet was washed with 70% ethanol and air dried. The pellet was dissolved in 8 ml of TE, pH 8.0.

For the caesium chloride ethidium bromide gradient, 8 gm of caesium chloride was added to the 8 ml of plasmid DNA solution. One ml of ethidium bromide at 10 mg/ml was added to this solution and the aggregates were removed by centrifugation at 3000 rpm for 30 min in an SS-34 rotor. The supernatant was loaded into a Beckman "Quick seal" polyallomer ultracentrifuge tube. The tubes were spun at 45,000 rpm in a Beckman VTi65 rotor for >16 hrs at 23°C. The plasmid band was removed from the tube by puncturing the side of the tube with a needle. Ethidium bromide was removed by extracting with butanol saturated with TE, pH 8.0. The aqueous solution was then transferred to a dialysis tubing and dialysed against TE, pH 8.0 to remove caesium chloride.

ii) Qiagen column method

In this method the DNA obtained by alkaline lysis was further purified by applying it on a column which retains DNA. After washing the column, bound DNA was eluted by increasing the salt concentration of the wash buffer.

A 400 ml of overnight culture was processed as described above, except 400μg DNase free RNase was added to the solution in place of lysozyme. The supernatant obtained after 5 M potassium acetate, pH 4.8
precipitation was loaded on a Qiagen-tip 500 prequillibrated with buffer QBT (750 mM NaCl, 50 mM MOPS, 15% ethanol, pH 7.0, 0.15% Triton X-100). The column was washed with 30 ml of buffer QC (1.0 M NaCl, 50 mM MOPS, 15% ethanol, pH 7.0). The plasmid DNA was eluted with 15 ml of buffer QF (1.25 M NaCl, 50 mM MOPS, 15% ethanol, pH 8.2). The eluted DNA was then precipitated with 0.7 volume of isopropanol and centrifuged at 5000 rpm for 30 min in an SS-34 rotor. The DNA pellet was washed with 70% ethanol and air dried.

**Plasmid DNA preparation-small scale**

For small scale preparation of plasmid DNA a 1.5 ml of overnight culture was used. These were then processed in the same way as the large scale plasmid DNA preparations except the volume of solutions were scaled down accordingly. Instead of proceeding to the caesium chloride gradient step the supernatant was extracted with phenol/ chloroform. The DNA was then ethanol precipitated, air dried and dissolved in 20 µl of TE, pH 8.0 containing 20 µg/ml of DNase-free RNase.

**Agarose gel electrophoresis of DNA**

Neutral agarose gels were made either in TBE or TAE (Table 2.1). Analytical gels were made by boiling 0.8-1.0% "Seachem" agarose (FMC, USA) in TBE whereas for preparative gel "Seaplaque" low gelling temperature agarose (FMC, USA) was used in TAE. The gels were run, unless otherwise mentioned, in the presence of 0.4 µg/ml ethidium bromide.

**Labelling of DNA**

The DNA used as a probe for Southern blotting was radioactively labelled by random hexamer labelling. Random hexamers (Pharmacia Fine Chemicals, Upsala, Sweden) were annealed with the DNA to be used as probe, at 90°C for 3 min and allowed to cool slowly at room temperature. This annealed mixture was then radioactively labelled with 25 Units of Klenow fragment of *E. coli* polymerase I in 50 mM Tris HCl, pH 7.5, 5 mM MgCl₂, 50 µg/ml BSA, 100 µM each of dGTP, dCTP, dTTP, 50 µCi α³²PdATP. The reaction was carried out at room temperature for 6-16 hrs. Unincorporated nucleotides were removed by passing the reaction mixture through Sephadex G-50 column. The wash-through fraction was
collected and 1 μl was used to measure the amount of radioactivity incorporated in the DNA.

**Southern blotting**

The DNA to be analysed by Southern blotting was digested with various restriction enzymes and depending on the size of the fragment to be analysed run on a 0.8-1.0% agarose gel. The gel was run at 50V for 6-8 hrs at room temperature. It was removed and incubated in depurination solution (Table 2.1) for 15 min. The gel was then rinsed three times with distilled water and incubated in denaturating solution and agitated for 15 min by that time the colour of bromophenol blue dye returns to its blue colour. The gel was again washed in distilled water and transferred to neutralising solution and agitated for 15 min.

The DNA from this agarose gel was transferred to Hybond N+ (Amersham, UK) by capillary blotting which was set up as follows: A glass dish was filled with 20xSSC (Table 2.1) and a supporting platform was made at the centre. It was covered with a wick made with Whatman 3MM filter paper saturated with 20xSSC. The gel was placed on the Whatman 3MM filter paper and was surrounded with parafilm. The air bubbles are removed with a pipette and a Hybond N+ membrane of the size of the gel was placed on the top of the gel. Three sheets of filter paper soaked in 20xSSC were placed on the top of the membrane and again air bubbles removed. A stack of absorbent paper towels was placed on the top of the membrane and a glass plate was placed over it. A weight of about 750 gm was applied and the stack was left for 4 hrs.

After the transfer the Hybond N+ membrane was washed with water and UV crosslinked for 3 min at a distance of 18 cm from the UV transilluminator. The membrane was prehybridised in prehybridisation buffer (Table 2.1) for 8 hrs at 65°C. Five millilitre of prehybrisation buffer was removed from the bag containing the membrane and appropriate amount of radiolabelled probe was added. This solution was added back to the container containing the Hybond N+ membrane and incubated overnight at 65°C.

The membrane was washed with 2XSSC containing 0.1% SDS for 30 min at 65°C. Depending on the signal to noise ratio the stringency of washes were increased to 0.1% SSC containing 0.1% SDS at 65°C. The blots were then autoradiographed at -70°C for appropriate length of time.
**Ligation of DNA**

Before ligation, DNA present in low melting agarose was heated to 70°C for 10 min and kept at 37°C until used for the reaction. Appropriate amount of DNA was mixed with the ligation buffer and DNA ligase (New England Biolabs, U.K). The ligation reaction was performed for 3 hrs at 25°C.

**Polymerase chain reaction**

The polymerase chain reaction was carried out in Perkin-Elmer-Cetus thermal cycler using Ampli Taq DNA polymerase (Cetus Corporation, USA). The reaction mixture contained from 1 ng to 1 μg of plasmid or genomic DNA in 10 mM Tris HCl, pH 8.3, containing 0.01% gelatin, 10% DMSO, 1.5 mM MgCl₂, 400 μM dNTP in a total volume of 100 μl. The primers at 750 nM were used in the reaction. The denaturation step was carried out at 95°C for 5 min and then 1.25 Units of Ampli Taq DNA polymerase was added. Each cycle consisting of a denaturation step at 92°C for 1 min, annealing at 45-55°C for 1 min and polymerisation at 72°C for 5 min was repeated 25 times. The amplified product was extracted with equal volume of chloroform/isoamylalcohol (24:1) before loading on agarose gel.

**2.4. BIOCHEMICAL TECHNIQUES**

**Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE)**

SDS-PAGE was performed according to the method of Laemmli, 1970. The recipes are given in Table 2.2. For protein analysis BioRad Mini Protean II electrophoresis apparatus was used. The running gel was cast vertically between two glass plates with a five percent stacking gel poured above it. The gel was run at 50-100V along with protein molecular weight markers. It was stained with coomassie brilliant blue for 5 min and destained until a clear background was obtained. The recipes for the coomassie brilliant blue is given in Table 2.2.

**Western blotting of SDS-PAGE separated proteins:**

The SDS-PAGE gel to be transferred was soaked in transfer buffer (see Table 2.2) for 15 min. A BA83 (0.2 μ) nitrocellulose membrane (Schleicher and Schuell, Germany) of the size of the gel presoaked in transfer buffer was layered over the gel. This was then sandwiched
between two Whatman 3MM filters and placed in the transfer apparatus containing transfer buffer. A current of 25 mA was applied for 1 hr. The transferred protein was detected on nitrocellulose by staining with Ponceau S (BDH, U. K.). After washing with phosphate buffered saline (PBS) (Table 2.3) the nitrocellulose membrane was blocked with 20% (w/v) dried skimmed milk for 1 hr at room temperature. Primary antibody was incubated for 4 hrs to overnight at 4°C. The filters were washed with PBS containing 1% NP40 for 30 min followed by alkaline phosphatase conjugated secondary antibody for 1 hr at room temperature. After washing the membrane with PBS containing 1% NP40 the substrates NBT/BCIP (Sigma Chemical Co.) was added for the detection of the bound secondary antibody conjugated to alkaline phosphatase.

**Protein Assay:**

Protein concentration was measured either at 280 nm where extinction coefficient was known or by the dye binding method of Bradford (1976). Eight hundred microlitre of protein solution containing between 0-50 μg/ml protein was treated with 0.2 ml of dye (BioRad, USA) and OD at 595 nm was read. The concentration of protein was calculated from the known standard of BSA (Fatty acid free, Sigma Chemical Co.) assayed along with the unknown sample.

**Colony blotting**

Bacterial colonies containing required expression plasmid was grown on L-agar plates containing appropriate antibiotic. The colonies were transferred to nitrocellulose membrane circles by layering the membrane on the top of the plate and peeling it off. It was then induced for 2 hrs on L-agar plate. The membranes were fixed in chloroform vapours for 30 min and processed as for the western blot.

**35S methionine labelling of cellular proteins**

Cells were grown to 75% confluency. The medium was drained off and replaced with 2 ml E4 medium (Table 2.5) without methionine containing 10% FCS. The cells were labelled with 250 μCi of 35S methionine per 10 cm plate for overnight. The cells were harvested with 5 mM EDTA in PBS and if not used immediately were frozen at -70°C.

**Preparation of soluble extracts**
Cell pellets were lysed in NET (Table 2.3) containing 1% NP40 and 1 mM PMSF. The lysed cells were passed several times through 27G needle to shear the chromosomal DNA and then centrifuged at 50,000xg for 30 min. The supernatant was removed and used.

Soluble extract (S100) from HeLa cells was made in hypotonic buffer (Table 2.3). The cell pellet was suspended in hypotonic buffer and homogenised with 10 strokes of dounce homogeniser. This was then left for 30 min on ice and centrifuged at 8,000 rpm for 30 min in a Sorvall RC-5B centrifuge using an SS-34 rotor. The salt concentration of the supernatant was adjusted to 0.1 M and centrifuged at 50,000 rpm for 30 min in TL-100 rotor in Beckman TL-100 Ultracentrifuge. The supernatant, if not used immediately, was stored at -70°C.

2.5 IMMUNOLOGICAL TECHNIQUES

Purification of monoclonal antibodies

Affi-prep protein A support (BioRad, USA) was used to purify monoclonal antibodies. Five millilitres of protein A beads were sequentially washed with 25 ml of 100 mM glycine HCl, pH 3.0, 25 ml of PBS, 25 ml of 50% methanol, 25 ml of PBS, 15 ml of 0.5 N NaOH, 25 ml of PBS, 50 ml of 50 mM sodium borate buffer containing 3.0 M NaCl, pH 8.9. The filtered tissue culture supernatant containing the relevant antibody in 0.1 M borate buffer, containing 3.3 M NaCl, pH 8.9 was loaded on the column. The column was washed with 10 column volumes of 3.0 M NaCl, 50 mM sodium borate, pH 8.9 and 10 column volume of 3.0 M NaCl, 10 mM sodium borate, pH 8.9. The bound immunoglobulin was eluted with 100 mM citrate buffer, pH 6.0 for IgG1 and IgG3, pH 5.0 for IgG2a and pH 4.0 for IgG2b.

Biotinylation of antibodies

Purified antibody (1-3 mg/ml) was dialysed in 0.1 M sodium borate buffer, pH 8.8 to remove sodium azide. Biotin N-hydroxysuccinimide (ICN Biomedicals, U. K.) was added to this antibody solution at a molar ratio of 20:1. The reaction was allowed to proceed for 4 hrs at room temperature. The reaction was stopped with ammonium chloride for 10 min at room temperature. The solution was then dialysed against PBS to remove uncoupled biotin.

Immunoprecipitation
Soluble cell extracts were pre-incubated with Protein G-Sepharose 4B for 30 min at 4°C. The solution was centrifuged at 3000 rpm for 5 min in an SS-34 rotor in Sorvall RC-5B centrifuge. The supernatant was incubated with equal volume of monoclonal antibody supernatant and incubated for at least 4 hrs at 4°C. Fifty microlitres of Protein G beads were added and incubated further for 30 min. The beads were washed five times with NET buffer supplemented with 250 mM NaCl, 1% NP40. The beads were then boiled in the SDS sample buffer for 5 min and loaded on 12.5% SDS-PAGE gels.

**Enzyme-linked immunosorbent assay (ELISA)**

PCNA used for the competitive ELISA was the soluble protein obtained from the plasmid construct pC10. One microgram per millilitre of the antigen was coated overnight on a 96 well microtiter plate. Nonspecific binding was blocked with 10% BSA in PBS for 3 hrs at room temperature. The plates were washed with 0.1% NP40 in PBS and 100 μl of biotinylated anti-PCNA (8 μg/ml) antibody was added. After 3 hr incubation, the plates were washed with 0.1% NP40 in PBS. Streptavidin-horseradish peroxidase (DAKO, Denmark) at a dilution of 1:1000 was added and incubated for 15 min at room temperature. The colour was developed using TMB and H₂O₂ and read at 450 nm on an ELISA reader after stopping the reaction with 50 μl of 1M H₂SO₄.

**Cell staining**

Petri dishes containing a monolayer of tissue culture cells were fixed with a 1:1 mixture of acetone and methanol for 5 min. The plates were then air dried. Monoclonal antibodies were then applied in 3 μl volume and incubated for 1 hr in a humidified chamber. After washing with PBS, 1:200 diluted horseradish peroxidase conjugated second antibody (DAKO, Denmark) was added and incubated for 1 hr at room temperature. The plates were washed with PBS and colour was developed with DAB and H₂O₂.

**2.6: MICROBIOLOGICAL TECHNIQUES**

**E. coli strains**

The *Escherichia coli* strains used were:
DH5α (Bethesda Research Laboratory, 1986): supE44 ΔlacU169 (ø80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1

BL21(DE3) (Studier and Moffat, 1986): hsdS gal(λcl857 ind1Sam7 nin5 lacUV5-T7 gene1).

N4830-1 (Gottesman et al., 1980): F-, su-, his-, ilv-, galKΔ8, Δ(chlD-pgJ), λ, Δ Bam, N+, cl857, ΔH1.

Y1090 (Huynh et al., 1985): F-Δ(lac)U169 lon-100 araD139 rpsL(strF) supF mcrA trpC22::Tn10 /pMC9 (lacIq,Tet'r,Amp')

Strains were grown in L-agar plates and stored by freezing at -70°C in 40% glycerol in L-broth.

**Culture Media**

Recipes of the culture media for *E. coli* are shown in Table 2.4.

**Antibiotics and supplements**

Ampicillin (Sigma Chemical Co. USA) was used at 100 μg/ml in liquid cultures and 50 μg/ml in plates.

Tetracycline (Sigma Chemical Co. USA) was used at 10 μg/ml both in liquid cultures and plates.

Chloramphenicol (Sigma Chemical Co. USA) was used at 25 μg/ml.

IPTG (Sigma Chemical Co. USA) was used for induction at a concentration of 500 μM.

**Transformation of *E. coli***

Competent *E. coli* cells were prepared by modification of the method of Mandel and Higa, (1970). An overnight culture was diluted 1:100 and grown until the OD at 600 nm of the culture reached 0.5. Fifty millilitre cell culture was centrifuged at 3000 rpm for 5 min in an SS-34 rotor in Sorvall RC-5B centrifuge and the pellet suspended in 50 ml of ice cold solution of 50 mM CaCl2. The cells were gently suspended and spun at 1000 rpm at 4°C in an SS-34 rotor. The pellet was suspended in 1.5 ml of 15%
glycerol in 50 mM CaCl$_2$. The cells were rapidly frozen in dry ice and stored at -70°C.

The cells to be transformed were thawed on wet ice and up to 1 ng DNA/50 μl of cells was added. After 30 min incubation on ice the cell were given heat shock at 37°C for 2 min. The cells were then diluted in 4 volume of SOB medium (see Table 2.4) and agitated for 1 hr at 37°C and plated on L-agar plate containing appropriate antibiotic.

**Plasmids used**

pUR288 (Ruther and Muller-Hill, 1983).

pRIT2T (Nilson *et al.*, 1985) was obtained from Pharmacia Fine Chemicals.

pT7.7 (Tabor and Richardson, 1985) was a gift from W. Studier, Brookhaven Laboratories, USA.

pEX2 (Stanley and Luzio, 1984) was obtained from Boehringer Mannheim.

pCR-1 (Matsumoto *et al.*, 1987) was a gift from Dr. T. Moriuchi, Tokai School of Medicine, Japan.

**Induction of *E. coli* for the expression of inducible protein**

Two types of induction methods were followed depending on the type of plasmid and host cell used.

i) Temperature induction

Heat induction was used for plasmid pRIT2T and pEX2 which had $\lambda PR$ promoter. An overnight culture was diluted 1:100 in L-broth and grown at 30°C to an OD at 600 nm of 0.5. The bacterial culture was induced by adding an equal volume of L-broth at 54°C to the culture and then incubated for 2 hrs at 42°C.

ii) IPTG induction

A bacterial culture was grown overnight at 37°C in presence of 100 μg/ml ampicillin. It was induced with 500 μM IPTG for 2 hrs. The cells were centrifuged at 3000 rpm for 5 min in an SS-34 rotor in Sorvall RC-
5B centrifuge. The pellet was stored at -70°C and processed for the purification of protein or boiled in sample buffer for SDS-PAGE.

2.7: CELL CULTURE

Cell lines and culture

CV-1 cells. African green monkey kidney cell line, were obtained from Dr. L.V. Crawford, ICRF.

HeLa cells. Transformed human epitheloid tumour cell line was obtained from ICRF stock.

Cell stocks were stored in 8% DMSO in FCS (GIBCO, USA) in liquid nitrogen. Cells were grown in E4 medium or RPMI-HEPES medium (Table 2.5) with 10% FCS. Cells were grown in plastic petri dish or flasks and gassed with 5-10% CO₂.

Culture media for fission yeast

The composition of the media used for growing fission yeast is given in Table 2.6.

Isolation of diploid and haploid strains of fission yeast

To check the ploidy of fission yeast, they were routinely streaked on a YEP plate (for recipe refer to Table 2.6). Phloxine B present in YEP plates accumulates in dead cells which stain dark red. Haploid strain were stained light pink and diploid colonies darker pink.

Preparation of S. pombe chromosomal DNA

The chromosomal DNA was prepared from S. pombe by the method of Durackcz et al., 1985. The cells were grown in 100 ml YE medium to early stationary phase at 30°C. The spheroplasts of these cells were made with Novozyme 234 (Novo Industri A/S, Denmark) in 50 mM citrate/phosphate, pH 5.6, 40 mM EDTA, 1.2 M sorbitol. After about 50% digestion of the cells they were centrifuged at 3000 rpm in an SS-34 rotor in Sorvall RC-5B centrifuge. The pellet was suspended in 15 ml of 50 mM Tris HCl, pH 7.5, 5 mM EDTA and 1.5 ml 10% SDS was added. To this 5 ml of 5 M potassium acetate was added and centrifuged at 5000 rpm for 15 min on an SS-34 rotor. The DNA in the supernatant was precipitated with isopropanol and treated with 20 µg/ml DNase free RNase. The DNA was
treated with phenol:chloroform and ethanol precipitated. The DNA was finally dissolved in TE, pH 8.0.

**Preparation of *S. pombe* RNA**

Total RNA from *S. pombe* was prepared essentially according to Maniatis *et al.*, 1982. The cells were grown to mid log phase (1 x10⁷ cells/ml). After centrifugation the cell pellet was suspended in 50 mM citrate, pH 5.6, 1 mM EDTA and 1 mM β-mercaptoethanol and digested with 1 mg/ml of Novozyme 234 for 30 min at 37°C. The pellet obtained after centrifugation at 3000 rpm in an SS-34 rotor was suspended in 4 M guanidine thiocyanate containing 1 mM EDTA. The solution was centrifuged again and the supernatant was loaded on a cushion of 5.7 M caesium chloride and centrifuged in a SW41 rotor at 32,000 rpm for 20 hrs. The pellet was washed with 70% ethanol and suspended in 10 mM Tris HCl, pH 7.5, 1 mM EDTA containing 0.2% SDS and ethanol precipitated. The pellet was washed with 70% ethanol and dissolved in DEPC treated water.
### TABLE 2.1: Buffers used in nucleic acid work

**Tris-EDTA pH 8.0 (TE pH 8.0)**

10 mM Tris HCl, 1 mM EDTA, pH 8.0.

**Tris-acetate-EDTA (TAE)**

40 mM Tris base, 5 mM sodium acetate, 1 mM EDTA, adjusted to pH 7.8 with acetic acid.

**Tris-borate-EDTA (TBE)**

89 mM Tris base, 178 mM boric acid, 2 mM EDTA.

**DNA gel loading dye**

10 mM EDTA, 10%(w/v) sucrose, 0.01%(w/v) bromophenol blue, 0.01%(w/v) xylene cyanol.

**Denaturing solution**

1.5 M NaCl, 0.5 N NaOH.

**Neutralising solution**

0.5 M Tris HCl, 1.5 M NaCl, pH 7.5.

**20xSSC (standard saline citrate)**

0.3 M sodium citrate, 3.0 M NaCl, pH 7.0.

**Prehybridization solution**

6xSSC, 0.01 M EDTA, 5x Denhardt's solution, 0.5%(w/v) SDS, 100 μg/ml denatured salmon sperm DNA

**50x Denhardt's solution**

1%(w/v) Ficoll, 1%(w/v) polyvinylpyrrolidone, 1%(w/v) BSA.
TABLE 2.2: Solutions used in SDS-PAGE and western blotting

**SDS-PAGE**

**Running gel:**

<table>
<thead>
<tr>
<th>Components</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%(w/v) acrylamide,</td>
<td>(1/10 x % of the running gel)x ml</td>
</tr>
<tr>
<td>1.33%(w/v) bisacrylamide</td>
<td>1.8 ml</td>
</tr>
<tr>
<td>1.0 M Tris HCl pH 8.8</td>
<td>20 μl</td>
</tr>
<tr>
<td>water</td>
<td>to 5 ml</td>
</tr>
<tr>
<td>10%(w/v) ammonium per sulphate</td>
<td>30-50 μl</td>
</tr>
<tr>
<td>TEMED</td>
<td>2-5 μl</td>
</tr>
</tbody>
</table>

**Stacking gel**

For 0.75 mm mini gel 1.5 ml of stacking gel was required

<table>
<thead>
<tr>
<th>Components</th>
<th>volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>50%(w/v) acrylamide</td>
<td>2.5 ml</td>
</tr>
<tr>
<td>1.33%(w/v) bisacrylamide</td>
<td>3.1 ml</td>
</tr>
<tr>
<td>1.0 M Tris HCl pH 6.8</td>
<td>125 μl</td>
</tr>
<tr>
<td>water</td>
<td>to 20 ml</td>
</tr>
<tr>
<td>10%(w/v) ammonium per sulphate</td>
<td>15 μl per gel</td>
</tr>
<tr>
<td>TEMED</td>
<td>1.5 μl per gel</td>
</tr>
</tbody>
</table>

**Sample buffer**

62.5 mM Tris HCl, pH 6.8, 100 mM DTT, 2%(w/v) SDS, 10%(v/v) glycerol, 0.01%(w/v) bromophenol blue.

**Stain**

0.25%(w/v) coomassie brilliant blue R-250, 40%(v/v) methanol, 7.5%(v/v) acetic acid.

**Destain**

40%(v/v) methanol, 7.5%(v/v) acetic acid.

**WESTERN BLOTTING**

**Transfer buffer**

25 mM Tris base, 192 mM glycine, 20%(v/v) methanol.

**Ponceau S**

2%(w/v) Ponceau S in 3%(w/v) trichloroacetic acid.

**Alkaline phosphatase buffer**

100 mM NaCl, 5 mM MgCl₂, 100 mM Tris HCl, pH 9.5.

**Substrate for alkaline phosphatase**

NBT: 5%(w/v) NBT in 70%(v/v) dimethylformamide
BCIP: 5%(w/v) BCIP in 100%(v/v) dimethylformamide

66 μl of NBT, 33 μl of BCIP was mixed in 10 ml of alkaline phosphatase buffer.
### TABLE 2.3: General buffers used in protein work

**Phosphate buffered saline (PBS)**
150 mM NaCl, 3 mM KCl, 8 mM NaH$_2$PO$_4$, 2.5 mM KH$_2$PO$_4$, pH 7.4.

**NET buffer**
150 mM NaCl, 50 mM Tris HCl, 5 mM EDTA, pH 7.5.

**Hypotonic buffer**
20 mM HEPES, pH 7.5, 1.5 mM MgCl$_2$, 5 mM KCl, 1 mM β-mercaptoethanol, 1 mM PMSF
TABLE 2.4: Bacterial culture and transformation media

L-broth
1% (w/v) Difco bacto-tryptone, 0.5% (w/v) Difco bacto-yeast extract, 1% (w/v) NaCl, adjusted to pH 7.5 with NaOH.

L-agar
L-broth containing 1.5% (w/v) Difco bacto-agar.

SOB medium
2% (w/v) Difco bacto-tryptone, 0.5% (w/v) Difco bacto-yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM MgCl₂, 10 mM MgSO₄.
**TABLE 2.5: Eukaryotic tissue culture media**

**E4 medium**
Dulbecco's modified Eagle's medium (DMEM) (prepared at Clare Hall) + 0.01% (w/v) streptomycin + 0.006% (w/v) penicillin (potassium salt) + antimycotic agents.
TABLE 2.6: Culture media for fission yeast

**Edinburgh minimal medium (EMM)**
14.7 mM potassium hydrogen phthalate, 15.5 mM Na$_2$HPO$_4$, 93.5 mM NH$_4$Cl, 111 mM glucose, 1 ml/litre vitamins (stock x 1000), 0.1 ml/litre minerals (stock x 10,000), 20 ml/litre salts (stock x 50).

**Salts x 50**
0.26 mM MgCl$_2$.6H$_2$O, 4.99 mM CaCl$_2$.2H$_2$O, 0.67 M KCl, 14.1 mM Na$_2$SO$_4$.

**Vitamins x 1000**
4.20 mM pantothenic acid, 81.2 mM nicotinic acid, 55.5 mM inositol, 40.8 mM biotin.

**Minerals x 10,000**
80.9 mM boric acid, 23.7 mM MnSO$_4$, 13.9 mM ZnSO$_4$.7H$_2$O, 7.40 mM FeCl$_2$.6H$_2$O, 2.47 mM molybdc acid, 6.02 mM KI, 1.60 M CuSO$_4$.5H$_2$O, 47.6 mM citric acid.

After autoclaving a few drops of preservative (1:1:2, chlorobenzene:dichloroethane:chlorobutane) was added.

**Minimal media**
EMM + 250 mg/litre supplements as required.

**Yeast extract (YE)**
0.5% (w/v) Oxoid yeast extract, 3.0% glucose.

**Yeast extract + supplements (YES)**
YE + 250 mg/litre adenine, histidine, leucine, uracil and lysine hydrochloride.

**Yeast extract + phloxine B (YEP)**
YES + 20 mg/litre phloxine B.
CHAPTER 3

CONSTRUCTION AND EXPRESSION OF PCNA

EXPRESSION PLASMIDS

3.1: INTRODUCTION

A cDNA encoding rat PCNA was obtained from Dr. Moriuchi (Matsumoto et al., 1987) and subcloned into several expression vectors. The objective was to obtain PCNA in soluble form either as a fusion protein or as an unfused protein. For this purpose the PCNA cDNA was subcloned in plasmid pRIT2T and pT7.7. β-galactosidase fusion proteins were also constructed with a full length as well as with fragments of PCNA cDNA.

There were two monoclonal antibodies against PCNA available while this study was being carried out (Ogata et al., 1987b). These antibodies were raised against denatured PCNA and did not recognise native protein. In order to understand the function of PCNA it was necessary to have immunological probes which would recognise the protein in its native form. For this reason the vector pRIT2T was selected as there were reports that the protein expressed in this vector was soluble (Nilson et al., 1985) and therefore did not need to be exposed to denaturing conditions during purification. This vector contains the λPr promoter and the first 12 codons of λcro/lacZ gene fused to a truncated form of the staphylococcal protein A gene. Downstream of this gene is a multiple cloning site (MCS) followed by transcription termination site. Translation termination sites are also present downstream of the MCS. The protein is expressed as a fusion protein fused at its N terminus to protein A. The fusion protein retains protein A activity and is therefore easily purified on an IgG-Sepharose column.

E.coli strain N4830-1 was used as the host cell for the plasmid pRIT2T. It carries the temperature sensitive λcl857 repressor. Temperature shift from 30°C to 42°C results in the derepression of the promoter by heat inactivation of the repressor, allowing initiation of transcription from the λPr promoter.
Another construct of PCNA was made in pT7.7 (Tabor and Richardson, 1985). This vector is based on the T7 polymerase system. The T7 RNA polymerase is very specific and will only transcribe genes that contain its own specific promoter sequence. Any gene placed under the T7 promoter is specifically transcribed when T7 RNA polymerase is present in the host cell. The vector pT7.7 contains the T7 010 promoter and the translational start site of gene10 protein. There is a multiple cloning region downstream of this start site. The host cell for this vector is BL21(DE3) which has the T7 RNA polymerase gene under the control of lacUV5 promoter, which is inducible by IPTG. This host cell is further modified by the introduction of the plasmid pACYC184, which encodes a T7 lysozyme gene and also 03.8 promoter for T7 RNA polymerase. T7 lysozyme is a natural inhibitor of T7 RNA polymerase and thus inhibits the basal level of T7 RNA polymerase activity. The expression of lysozyme depends on the orientation of the T7 lysozyme gene. If the lysozyme gene is in the right orientation and transcribed from the tet promoter of pACYC184, the expression of lysozyme is high and this strain is referred to as pLysE. The expression of lysozyme is low when the lysozyme gene is in reverse orientation and this strain is called pLysS. The choice of the cells depends on the toxicity of the cloned gene.

A construct expressing a β-galactosidase PCNA fusion protein was made in pUR288 (Ruther and Muller-Hill, 1983). This vector has a multiple cloning site at the 3' end of the lacZ gene which results in the expression of the fusion protein as a part of β-galactosidase. The induction by IPTG results in the production of large amount of protein. Since there are few bacterial proteins of the size of β-galactosidase, the fused proteins are easily identified by SDS-PAGE.

Another vector producing a β-galactosidase fusion protein pEX2 (Stanley and Luzio, 1984) was also used in this study. This vector contains a λ cro.E.coli lacZ gene under the control of λ Pr promoter. E. coli strain N4830-1 was used as the host cell for this plasmid.

### 3.2: CONSTRUCTION OF PLASMIDS

**pC288**

The rat PCNA cDNA cloned into PstI site of the plasmid pBR322, designated pCR-1 was obtained from Dr. T. Moriuchi. It was subcloned in β-galactosidase vector, pUR288. For this the vector was digested partially...
with EcoRI and filled with Klenow fragment and redigested with XbaI. The plasmid pCR-1 was digested with XbaI and HincII, which resulted in two fragments of 1.0 and 1.1kb. The purified 1.0 kb fragment containing the complete PCNA cDNA was ligated into the XbaI and blunt ended EcoRI site pUR288 to give pC288 (Figure 3.1). This construct contains 23 nucleotide from the 5' untranslated region of PCNA. *E. coli* strain DH5α was transformed with this vector.

The ampicillin resistant transformants were analysed by checking for the presence of the insert. Cell extracts were then prepared from the positive colonies and analysed by SDS-PAGE to determine whether an inducible fusion protein was produced (Figure 3.6).

**pCNA2T**

A protein A-PCNA fusion protein was made in protein A expression vector pRIT2T (Nilson *et al.*, 1985). A 728 bp fragment obtained after digestion of pC288 with *BamH*I and *PstI* was subcloned between *BamH*I and *PstI* site of the pRIT2T multiple cloning site (Figure 3.2). The PCNA cDNA cloned in this vector lacked the 3' end 93 nucleotides. This construct was transformed into *E.coli* strain N4830-1.

Colonies containing the insert were induced by temperature shock from 30°C to 42°C and protein analysed by SDS-PAGE (Figure 3.7). The IgG binding activity of protein A was exploited in the detection of the fusion protein. A protein band of apparent molecular weight of 65 kd was observed with alkaline phosphatase conjugated rabbit immunoglobulin on a western blot of the total cell extract. Since the molecular weight of protein A in the fusion protein was 27 kd, the expected size of the fusion protein should be 54 kd, instead of 65 kd. The apparent increase in the size of the fusion protein could be due to two reasons. First, the PCNA cDNA was incomplete at its 3' end and therefore lacked a translational termination signal. The translation termination signal of the vector was used to terminate the translation of the fusion protein. Therefore the actual size of the product would be greater than the expected molecular weight. Secondly, PCNA shows an anomalous molecular weight on SDS-PAGE (Matsumoto *et al.*, 1987). The calculated molecular weight of PCNA is 29 kd but it migrates on SDS-PAGE as a protein of 36 kd.

**pC10**
For cloning PCNA cDNA in pT7.7, the vector was digested with HindIII, made blunt-ended by the Klenow fragment and cut again with BamHI. This was ligated with a 946 bp BamHI and EcoRV fragment of pC288 to give pC10 (Figure 3.3). E. coli strain DH5α were transformed with this construct.

The colonies containing the insert were identified by analysing the plasmid DNA. E. coli strain pLysS BL21(DE3) were then transformed with the plasmid DNA containing the insert. The expression of protein was induced by IPTG. PCNA expressed in these cells could be identified by comparing the pattern of the coomassie blue stained protein bands by one dimensional SDS-PAGE of the cells containing pC10 and pT7.7 (Figure 3.8). The identification of the unique protein band present in pC10 as PCNA was further confirmed by western blotting the total cell extract with a mouse polyclonal antiserum raised against the protein A-PCNA fusion protein.

PC10-M

A full length rat PCNA cDNA was cloned into the plasmid pT7.7 such that the translation started from the first amino acid of the PCNA cDNA.

For subcloning in pT7.7, the plasmid was digested with NdeI and SmaI. A pair of oligonucleotide primers were synthesized to amplify the PCNA cDNA such that there was no untranslated region at the 5' and 3' ends. An NdeI site was incorporated in primer P1 and a SmaI site in primer P2. The sequence of the primer used are shown below with the restriction sites underlined.

P1: AACTCGCCCATATGTGGAGGCACGC

P2: TTCTAGCCCGGGTTAAGATCCTTCTTTC

After amplification of PCNA cDNA by PCR using primers, P1 and P2 the amplified product was digested with NdeI and SmaI and ligated into pT7.7 digested with the same restriction enzymes. DH5α were transformed with this ligation mixture. After checking for the presence of the insert, the plasmid DNA from the positive colonies were selected and pLysS BL21(DE3) cells were transformed with it.

pEX2-PCNA
The β-galactosidase expression plasmid pEX2 was used to express various fragments of PCNA for epitope mapping. The fragments of PCNA were made using the PCR. The primers have an EcoRI site at 5' end and BamHI site at 3' end and are shown in Table 3.1. A translation termination codon was placed at the end of the desired fragment to avoid read-through into the vector. The size of the translated fragment of PCNA is shown in Figure 3.5. β-galactosidase fusion protein containing (i) PCNA fragment P1 corresponding to amino acid 1-204 was amplified using primers A and B (Table 3.1), (ii) PCNA fragment P2 corresponding to amino acid 1-136 was amplified using primers A and C, (iii) for PCNA fragment P3 corresponding to amino acid 1-101, primers A and D were used and (iv) for PCNA fragment P4, corresponding to amino acid 1-68, primers A and E were used. After amplification the product was digested with EcoRI and BamHI and ligated into the EcoRI and BamHI fragment of the vector pEX2 (Figure 3.4). E. coli strain N4830-1 were then transformed with this construct.

The bacterial colonies containing the inserts were identified by colony blot screening using a mixture of anti-PCNA monoclonal antibodies. The colonies showing positive reaction were further analysed by running a total cell extract on SDS-PAGE. Various fragments of PCNA expressed as β-galactosidase fusion protein prepared in this way are shown in Figure 3.5 and 3.10. The presence of insert was also confirmed by restriction enzyme analysis.

3.3: EXPRESSION AND PURIFICATION OF PROTEIN FROM BACTERIAL CONSTRUCTS

Solubility of pC288

A 152 kd protein band of β-galactosidase-PCNA fusion protein was produced when DH5α cells harbouring the recombinant plasmid were induced with IPTG. Since a large amount of the fusion protein was produced, it appeared to be a good source for use as an antigen to raise antibodies against PCNA. For this purpose the solubility of this protein was studied. A bacterial culture was induced as described before. The cells were lysed in 50 mM Tris HCl, pH 7.5, 10 mM EDTA containing 1 mg/ml lysozyme. The 50,000 x g supernatant and pellet were analysed on SDS-PAGE. The fusion protein was in the insoluble pellet. Increasing the salt concentration did not have any influence on its solubility.
An inclusion body preparation containing the fusion protein was prepared and solubilised in 8 M urea. The protein became insoluble as soon as the urea concentration was reduced to 3 M. Thus a soluble protein could not be obtained from this construct and therefore an alternative source of PCNA was looked for.

**Purification of protein A-PCNA fusion protein**

The expression of the protein A-PCNA fusion protein is induced by increasing the temperature of the culture. The amount of protein induced depend on several factors. One important factor was found to be the rapidity of the temperature shift. To achieve this L-broth was pre-warmed to 54°C and then an equal volume of this was added to the culture grown at 30°C so that the final temperature of the mixed culture was 42°C. A time course of induction performed in this manner showed that the maximum protein A-PCNA fusion protein was obtained 90 min to 120 min after the temperature shift. Induction beyond this period led to the degradation of the fusion protein. In a typical large scale preparation an overnight culture grown at 30°C was diluted 100 fold and allowed to grow till the OD reached between 1.0-2.0. An equal volume of culture medium at 54°C was added to this diluted *E. coli* strain N4830-1 culture harbouring the relevant plasmid and the induction was allowed to proceed for 2 hr at 42°C.

The solubility of the protein A-PCNA protein was also studied. The cells were lysed in PBS containing 1 mg/ml lysozyme. After sonication the cell lysate was centrifuged at 30,000 rpm in 60Ti rotor in Beckman L8-55M ultracentrifuge for 30 min. The protein A-PCNA fusion protein in the supernatant and pellet fractions were detected by western blotting using alkaline phosphatase conjugated rabbit antibody. The protein A-PCNA fusion protein was found to be soluble under these conditions.

For the purification of the protein A-PCNA fusion protein the cells were pelleted at 7000 rpm in a GSA 600 rotor in Sorvall RC-5B centrifuge for 15 min. The pellet was suspended in PBS and soluble lysate prepared as described above. This was applied on a column of human IgG-Sepharose 6 Fast Flow (Pharmacia Fine Chemicals, Uppsala, Sweden) pre-equilibrated with PBS. The column was washed with 50 column volumes of PBS containing 1 M NaCl and 0.1% NP40 and the bound protein was eluted with 100 mM glycine HCl, pH 3.0. The fractions were neutralised immediately with 2 M Tris base. A yield of 1-2 mg fusion protein/400 ml starting culture was obtained.
Purification of PCNA protein from *E. coli* harbouring the plasmid pC10

The PCNA protein encoded by the plasmid pC10 had an additional 20 amino acid at its N terminus derived from the amino terminus of bacteriophage T7 gene 10 protein. The expression of the fusion protein was induced by IPTG. Time course studies showed that the maximum induction was achieved within 2 to 3 hrs after which the protein started to degrade. It was completely degraded after 10 to 16 hrs of induction. Another factor that was found to be important was the OD at 600 nm at which the cells were induced. An OD at 600 nm of 0.5-0.7 gave a maximum expression of the protein when induced for 2 hrs at 37°c.

The protein produced by the plasmid construct pC10 was detected first by SDS-PAGE of the total cell lysate of cells with and without the plasmid containing the PCNA cDNA insert. This was further confirmed by western blotting the extract using a mouse polyclonal antibody against PCNA. The solubility of this protein was also investigated. Since the host cell contained a plasmid expressing lysozyme, the cells were lysed by freezing and thawing which disrupted the cell membrane and released the enzyme. The supernatant and pellet were analysed for the presence of PCNA. The PCNA fusion protein expressed by pC10 was found to be present in the pellet fraction. Increasing the salt concentration did not affect the solubility of the PCNA. The protein was insoluble in 2 M urea and the solubility increased to 100% in 8 M urea.

PCNA expressed by the plasmid construct pC10 was purified from the inclusion bodies. The pLysS BL21(DE3) cells containing pC10 was induced as described above. The cell pellet obtained at 7000 rpm (GSA 600 rotor in a Sorvall RC-5B centrifuge) was rapidly frozen. The cells were thawed in 50 mM Tris HCl, 10 mM EDTA, pH 8.0. After sonication the lysate was centrifuged at 30,000 rpm in 60Ti rotor in Beckman L8-55M centrifuge. The pellet was washed with 1% NP40 to remove contaminant proteins. The pellet was further washed in 1 M urea and dissolved in 8 M urea. This solution was gradually dialysed against PBS. The dialysed protein remained soluble in PBS.

**Solubility of pC10-M**

To see whether the untranslated region had any effect on the solubility, a plasmid construct was prepared in which there was no other amino acid either from the T7 gene 10 protein of the vector or from the 5'
end of the PCNA cDNA. The lysS BL21(DE3) cells containing this plasmid were induced in the similar manner as pC10. The cells were pelleted and rapidly frozen. They were lysed in 50 mM Tris HCl, 10 mM EDTA, pH 8.0. The lysate was sonicated at maximum speed for 20 sec and then centrifuged at 30,000 rpm in 60Ti rotor in Beckman L8-55M centrifuge. The supernatant and pellet fractions were analysed by SDS-PAGE and western blotting and are shown in Figure 3.9. Monoclonal anti-PCNA antibodies were used to detect the expressed PCNA in these fractions. Although the expression of the protein was lower as compared to that obtained with plasmid pC10 the expressed protein was present only in the soluble fraction.

3.4: DISCUSSION

In this chapter the preparation of various constructs designed to express PCNA in *E. coli* during the course of this study have been described.

The fragment of PCNA cDNA subcloned into pRIT2T encoded amino acid 1-231. There were two advantages of subcloning in this vector. First the protein was in the soluble fraction of the lysate. Secondly, since it had protein A attached to the N- terminus of the protein it could easily be detected by using alkaline phosphatase conjugated rabbit immunoglobulin. Purification was essentially a one step process by affinity chromatography using IgG-Sepharose column. Furthermore, this construct could also be used in various biochemical experiments and the presence of PCNA could be monitored by using alkaline phosphatase conjugated rabbit immunoglobulin as a probe.

The protein eluted from the IgG-Sepharose column was about 95% pure as judged by SDS-PAGE. There was a protein band of about 70 kd that co-purified with the protein A-PCNA. This protein appeared to be associated with the protein A moiety rather than PCNA since the same molecular weight protein appeared in the fractions containing only protein A. Since the induction was by temperature shift one of the possibility might be an association of heat shock protein. However, western blotting with the polyclonal antisera against 70 kd heat shock protein, dnaK, did not give any reaction arguing against this hypothesis.

To obtain PCNA without fusion to another large protein PCNA cDNA was subcloned in pT7.7. This construct had 20 amino acids additional from the N terminus of bacteriophage T7 gene 10 protein. Full length PCNA was
subcloned in this construct. Since the protein was insoluble in PBS the purification was achieved by selective washing of the inclusion bodies and solubilising them. Protein produced by pC10-M was found to be soluble in 50 mM Tris HCl, 10 mM EDTA, pH 8.0. Thus a source of full length PCNA was obtained which would be useful for biochemical and functional analysis.

Although the β-galactosidase-PCNA fusion protein was insoluble, this construct was found useful in the epitope mapping of the antibodies. More constructs were made by deleting various fragments of PCNA by PCR. The advantage of using PCR is that the deletion could be made at specific regions and read-through into the vector could be avoided by introducing a translational termination codon. The fragments of PCNA expressed as β-galactosidase fusion proteins are listed in Figure 3.5.

Thus the initial objective of obtaining a relatively large amount of soluble PCNA was achieved. Also a construct containing a full length rat PCNA was made. PCNA expressed from the constructs pCNA2T and pC10 were used as an immunogen for raising the monoclonal antibodies described in the next chapter. The β-galactosidase-PCNA fusion protein together with the PCNA fusion protein expressed from pC10 was used to characterise these anti-PCNA monoclonal antibodies.
Figure 3.1: Diagrammatic representation of pC288. Relevant restriction enzyme sites are indicated.
Figure 3.2: Diagrammatic representation of pCNA2T. The restriction enzyme sites used for cloning rat PCNA cDNA in pRIT2T are indicated.
Figure 3.3: Diagrammatic representation of pC10. The restriction enzyme sites near the PCNA cDNA are indicated. The abbreviations used are: $\varnothing 10$, T7 RNA polymerase promoter; rbs, ribosome binding site.
Figure 3.4: Diagrammatic representation of pEX2. The fragments of rat PCNA cloned in EcoRI and BamHI sites are shown in Figure 3.5.
Figure 3.5: Diagrammatic representation of full length and various fragment of PCNA cloned in pEX2. The length of the fragments are represented by amino acid numbers given in brackets.
Figure 3.6: SDS-PAGE of the total cell lysate containing pC288. Total cell extract of DH5α containing, lane 1: pUR288; lane 2: pC288.
Figure 3.7: SDS-PAGE and western blot analysis of pCNA2T. Total cell extract of *E. coli* strain N4830-1 containing, lane 1 & 3: pRIT2T, lane 2 & 4: pCNA2T; Lane 3 & 4 are the western blots of the total cell extracts probed with alkaline phosphatase conjugated rabbit immunoglobulin.
Figure 3.8: SDS-PAGE of total cell extract of pLysS BL21(DE3) containing pC10. Lane 1: pT7.7; lane 2: pC10; Lane 3: Purified protein from pLysS BL21(DE3) containing pC10.
Figure 3.9: Western blot analysis of total cell extract of plysS BL21(DE3) containing pC10-M. Lane 1: total HeLa cell extract, lane 2: total cell extract of pLysS BL21(DE3) containing pT7.7; lane 3: pC10-M; lane 4: pellet fraction from lane 3 and lane 5: supernatant fraction from lane 3. The monoclonal antibody PC10 was used for western blotting.
Figure 3.10: SDS-PAGE of total cell extract of *E. coli* strain N\(^{4}830-1\) containing various fragments of PCNA in pEX2. Lane 1: pEX2; lane 2: pEX2.P1; lane 3: pEX2.P2; lane 4: pEX2.P3; lane 5: pEX2.P4.
### TABLE 3.1: Primers used in the amplification of fragments of rat PCNA

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<tr>
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80
CHAPTER 4

PRODUCTION AND CHARACTERISATION OF MONOCLONAL ANTIBODIES AGAINST PCNA

4.1: INTRODUCTION

In order to understand the function of PCNA it was necessary to have mono-specific antibodies that would recognise the native protein. There were two sources of antibodies against PCNA that were available while this study was being carried out. Sera from some of the patients suffering from SLE, an autoimmune disease, contains antibodies against PCNA (Miyachi, 1978). One of these autoimmune sera, AK, recognises native protein and inhibits DNA replication (Tan et al., 1987, Zuber et al., 1989). However, these sera are not mono-specific and contain antibodies against other nuclear proteins. It was also difficult to get sufficient amount of mono-specific sera against PCNA because of the low incidence of lupus patients and the low incidences of the presence of antibodies against PCNA in these patients. There were also two monoclonal antibodies, 19A2 and 19F4, raised against rabbit PCNA (Ogata et al., 1987b). Since SDS denatured antigen was used in those studies, these monoclonal antibodies neither recognised native PCNA nor inhibited DNA replication. However, they were able to recognise PCNA from various other mammalian species. They also recognised proliferating cells in tissues (Garcia et al., 1989). Since there was no good immunological reagent available which was mono-specific and recognised PCNA in its native conformation we decided to raise monoclonal antibodies against PCNA.

The best source of antigen for the production of antibodies against PCNA would be that purified from mammalian tissues. But the complexity of the purification procedure and relatively poor yield compelled us to look for an alternative source. Therefore, rat PCNA cDNA was expressed as a fusion protein in E. coli and the soluble protein obtained was used as an antigen to raise monoclonal antibodies against PCNA.

4.2: METHODS AND MATERIALS
**Immunization of mice**

The protein A-PCNA fusion protein was used to immunise six Balb/c mice. Fifty micrograms of the protein A-PCNA fusion protein in 100 μl of PBS was emulsified with an equal volume of Freund's complete adjuvant and injected intraperitonially into each mouse. This dose was repeated three times, at 21 days interval. The titre of the antibody was monitored by western blotting. The protein A-PCNA fusion protein was a good antigen for two reasons. First it was soluble and secondly, it had protein A attached to it which might enhance immunogenicity. The main drawback with this protein A-PCNA fusion protein is that protein A itself is very immunogenic and thus antibodies against it would also be raised. To minimise this one of the mice, having the highest titre of the antibody as judged by western blotting, was given a rest of three months. The mouse was then boosted with the full length PCNA produced from the plasmid pC10. Ten micrograms of this protein was injected on two successive days: once intraperitonially and once intravenously. Three days later the mouse was killed and spleen removed for fusion.

**Monoclonal antibody production**

The spleen cells were fused to the myeloma cells Sp2/0-Ag14 using 50% polyethylene glycol 1500. The cells were grown in 20% FCS in E4 medium in presence of the growth factor interleukin 6 at 4 ng/ml. No feeder cells were used. Hypoxanthine (1.0 μM) and azaserine (5.8 μM) were added to the medium for the selection of the hybridoma cells. After testing the supernatant by cell staining using CV-1 cells, the hybridoma cells showing positive nuclear staining were single cell cloned. The presence of selection and the growth factor were maintained throughout the single cell cloning.

Single cell cloning of hybridoma cells was achieved by the method of limiting dilution. Fifty microlitre of tissue culture medium containing about 50 hybridoma cells were transferred to the top left well of a 96 well tissue culture plate. This suspension was doubly diluted in E4 medium containing 20% FCS and interleukin 6 (4 ng/ml) across the plate. Each well across the plate was again doubly diluted along the plate. The cells were checked for antibody production after 10-15 days of plating. This procedure was repeated once more to ensure the clonability of the monoclonal antibody producing hybridoma cells.
Isotyping of the monoclonal antibodies

The monoclonal antibodies were isotyped using an Isotyping Kit for Mouse Monoclonal Antibodies (Serotec, Oxford, UK). The kit contained sheep red blood cells coated with isotype specific antibody. These cells were mixed with the monoclonal antibody. Agglutination occurred when the coated cells recognised the particular isotype of the antibody against which they were directed. The agglutinated cells settled at the bottom of the round bottom tissue culture well and were easily identified from the unagglutinated cells.

4.3: CHARACTERISATION OF THE MONOCLONAL ANTIBODIES

Eleven monoclonal antibodies against PCNA were produced and were named PC1 to PC11. They were characterised by various methods. Their properties are summarised in Table 4.1.

Western blotting

One of the methods used to screen the hybridoma supernatant was western blotting. All the eleven antibodies recognised PCNA in the soluble extract of HeLa cells (Figure 4.1) albeit with different efficiency. The monoclonal antibodies PC3 and PC6 reacted very strongly whereas the reaction with PC7 was very weak. They all recognised the 36 kd PCNA band but two antibodies, PC2 and PC10 recognised an additional protein band of 120 kd.

Immunoprecipitation

The ability of the monoclonal antibodies to immunoprecipitate PCNA from a $^{35}$S methionine labelled extract of CV-1 cells was also checked. With the exception of PC11 all the monoclonal antibodies recognised native PCNA in this immunoprecipitation assay. Substantial amounts of PCNA were immunoprecipitated with PC1, PC2, PC3, PC7, PC8 and PC10 whereas very little was immunoprecipitated with PC4, PC5, PC6 and PC9 (Figure 4.2). The identity of the 36 kd protein band as PCNA was confirmed by western blotting the immunoprecipitates with anti-PCNA mouse polyclonal antibodies. Anti $\beta$-galactosidase monoclonal antibody, BG2 was used as a control.

In these immunoprecipitation experiments two polypeptides coprecipitated along with PCNA. They had molecular weights of 45 kd and 25 kd. To rule out that the 25 kd polypeptide band might be the degradation
products of PCNA this was western blotted with a mixture of anti-PCNA monoclonal antibodies and mouse polyclonal serum. This band failed to give any signal with the anti-PCNA monoclonal and mouse polyclonal antibodies.

An affinity column was prepared using a mixture of five anti-PCNA monoclonal antibodies, PC1, PC2, PC3, PC8 and PC10. These monoclonal antibodies were chosen because they were able to immunoprecipitate substantial amounts of PCNA from 35S methionine labelled extracts. SDS-PAGE analysis of the proteins eluted from this column is shown in Figure 4.3. Beside 36 kd PCNA protein band four other distinct protein bands were observed. The apparent molecular weight of these bands were 45, 25, 20 and 18 kd. The 45 and 20 kd polypeptides were not detected in the immunoprecipitates when the gel was stained with coomassie blue as they migrated very close to the heavy chain and light chains of the antibody. The 18 kd polypeptide seem to be associated tightly with PCNA and is not dissociated at 600 mM NaCl concentration. On the other hand the 25 kd polypeptide was removed from the complex at 250 mM salt concentration. Washing the immune complex with nonionic detergent, e.g., 0.1% NP40 did not disrupt the association of these polypeptide chains to PCNA.

ELISA

i) Competitive ELISA

In order to see if the eleven antibodies raised against PCNA recognise stearically different epitopes competitive and sandwich ELISA were performed. For the competitive ELISA four of the monoclonal antibodies (PC3, PC8, PC9 and PC10) were biotinylated. The binding of these antibodies to the antigen was then quantitated by measuring the OD at 450 nm in the presence of unlabelled monoclonal antibodies. The results are summarised in Table 4.2 and shown in Figure 4.4, 4.5 and 4.6. The eleven antibodies can be divided into two main groups: competing and non-competing. The monoclonal antibodies PC1, PC2, PC3, PC5, PC6, PC8 and PC10 fall in one group. All of them compete with each other although with different efficiencies. Monoclonal antibodies PC4, PC7 and PC9 did not compete with rest of the monoclonal antibodies.

ii) Sandwich ELISA

Sandwich ELISA was performed to see whether PCNA captured by one antibody could be recognised by other monoclonal antibodies. For this, biotinylated PC9 antibody was used since it falls in the group of non-
competing monoclonal antibodies. PC9 was able to recognise PCNA captured by PC2, PC3, PC5, PC6, PC8, PC10 and PC11. It did not detect PCNA captured by PC1, PC4 and PC7. These results are shown in Figure 4.7.

Immunofluorescence studies

Previous reports of the immunofluorescence analysis of asynchronously growing cultured cells showed dramatic changes in the localisation of PCNA during the cell cycle (Celis and Celis, 1985). When organic solvents were used as fixative the immunofluorescence staining was only observed in the cells in the S phase of the cell cycle. Cells in G1, G2 and M phase exhibited weak staining with anti-PCNA antibodies. In the early S phase PCNA was localised in the nucleoplasm. This staining gradually increased as the cell progressed through the S phase. Before the maximum DNA synthesis PCNA redistributed to show punctate staining which at the stage of maximum DNA synthesis co-localised with the nucleoli. In the late S phase the staining gradually became nucleoplasmic and faded away.

When the anti-PCNA monoclonal antibodies were used to stain CV-1 cells, with the exception of the monoclonal antibodies PC4, PC7 and PC9, all the monoclonal antibodies showed the same immunofluorescence pattern as had been discussed above (Figure 4.8A). Nuclear staining was observed which showed variation from cell to cell. Amongst the three antibodies, PC7 did not show any reactivity in immunofluorescence. Monoclonal antibody, PC4 had a markedly different immunofluorescent staining pattern than had been observed with polyclonal and monoclonal antibodies against PCNA. The nuclear staining was very granular which in others was uniformly distributed, whereas in some cells the staining appeared to exclude the nucleoli (Figure 4.8C). Monoclonal antibody, PC9 showed only nucleolar staining. About 90% cells showed this type of staining pattern with PC9 and the rest of the cells did not show any nuclear staining (Figure 4.8B).

Tissue staining

Since antibodies against PCNA had previously been used as proliferation marker, these monoclonal antibodies were used to check for their reactivity on formalin fixed paraffin embedded tissue sections. The monoclonal antibodies PC2, PC5, PC6, PC8, PC10 and PC11 stained the nuclei of the germinal centres of the tonsil sections (Figure 4.9A & B).
Some of these antibodies also showed reactivity on sections of rectal carcinoma tissue. The antibodies PC2, PC10 and PC11 stained the proliferating cells in these sections (Figure 4.9C). Antibody PC10 showed strongest reactivity on the tissue sections.

**Immunoreactive forms of PCNA in *S. pombe* and in insect cells detected by monoclonal antibodies**

Since HeLa and CV-1 cells were used to screen the monoclonal antibodies, we wanted to know whether these antibodies would also recognise PCNA from different species. Therefore, extracts from *spodoptera frugiperda* (insect cells) and *S. pombe* were used in western blotting to see the immunoreactivity with these monoclonal antibodies. The monoclonal antibodies PC2, PC3, PC5, PC8 and PC10 gave a positive reaction on western blot (Figure 4.10 and Figure 4.11). PC10 was particularly strong in these reactions. These monoclonal antibodies were also checked for their reactivity with PCNA from *S. cerevisiae*. None of the monoclonal antibodies recognise PCNA in total cell extracts of *S. cerevisiae* or in *E. coli* over-expressing *S. cerevisiae* PCNA.

**Epitope mapping of anti-PCNA antibodies**

Various constructs of rat PCNA were made in the plasmid pEX2 and used to map the epitopes of the monoclonal antibodies against PCNA. The preparation of the constructs are described in Chapter 3. Various regions of cDNA were deleted from the 3'end. The reactivities of the monoclonal antibodies to these constructs were analysed by western blotting and the results obtained are shown in Figure 4.12. Since PC7 was very weak on a western blot it could not be mapped by this method.

From Figure 4.12 it can be seen that monoclonal antibodies PC2, PC3, PC5, PC8 and PC10 react with amino acid 1-136 of rat PCNA and amino acid 83-261 of *S. pombe* PCNA. Therefore their epitopes must lie between amino acid 83-136. None of these monoclonal antibodies reacted with a PCNA fragment containing amino acid 1-101 of rat PCNA despite abundant expression of this protein as seen by coomassie staining of SDS-PAGE gels. It is probable that the epitopes of these monoclonal antibodies lie between 101-136. Monoclonal antibodies PC1, PC4, PC6 and PC11 did not show any reaction with the PCNA fragment containing amino acid 1-101 but reacted with PCNA fragment containing amino acid 1-136. Therefore the epitopes of this group of monoclonal antibodies must lie between 1-136,
probably between 101-136. Monoclonal antibody PC9 reacted with the PCNA fragment containing amino acid 1-204 but did not react with amino acid 1-136. Therefore the epitope of this antibody lies between 1-204 probably between 137-204. This is also consistent with the result obtained with ELISA in which the antibody PC9 did not compete with any other monoclonal antibodies.

4.4: DISCUSSION

Eleven monoclonal antibodies were raised against rat PCNA using a fusion protein between protein A and PCNA. All the antibodies recognise PCNA on a western blot and ten of them were able to recognise native PCNA in an immunoprecipitation assay.

Four PCNA associated proteins were identified while analysing the immunoprecipitation of PCNA using these monoclonal antibodies. It would be interesting to see if these PCNA associated proteins have any effect on DNA replication.

The majority of the anti-PCNA monoclonal antibodies recognise epitopes in the region 101-136. The monoclonal antibody, 19A2, raised previously also maps within this region, i.e., between amino acid 111-125 (Huff \textit{et al.}, 1990). This monoclonal antibody was mapped by ELISA using synthetic peptides. It is interesting to note that the experimentally induced antibodies are mostly directed against primary sequence, denaturation resistant epitopes, whereas anti-PCNA antibodies present in SLE patients are directed against conformation dependent epitopes. When the epitopes of these autoimmune sera were mapped using synthetic 15 mer peptides in ELISA they fail to show any reaction. Their epitopes could only be mapped by immunoprecipitating the \textit{in vitro} translated fragments of PCNA cDNA. Some of the autoimmune antibodies fail to recognise PCNA protein on western blots. Most of the antibodies, experimental or otherwise, are directed against the region consisting of amino acid 60-150. It seems likely that this region is exposed in the PCNA molecule and plays an important role in maintaining the tertiary structure of the protein.

Immunofluorescence analysis of PCNA using monoclonal antibodies shows that the antigen is present in the nucleoplasm and goes to the nucleolus in mid S phase when DNA synthesis is maximum (Bravo and Macdonald-Bravo, 1985). However, the monoclonal antibody, PC9 which maps to amino acid 137-204, shows that PCNA is present in the nucleolus for the majority of the cell cycle. It is conceivable that majority of the
antibodies, since they map to the central region of PCNA molecule, are only able to recognise a subpopulation of PCNA. It seems that PCNA is present in nucleolus associated with some other protein(s). This associated protein(s) probably dissociate during the mid S phase and exposes the epitopes recognised by the majority of the other anti-PCNA antibodies. The question then arises what is PCNA doing in the nucleolus? Several replication proteins have been reported to be involved in transcription (DePamphilis, 1988). Since the concentration of PCNA seem to be in excess of that required during replication it is possible that PCNA may have some other nonreplicative role in the cell.

The monoclonal antibodies PC2, PC5, PC6, PC8, PC10 and PC11 are useful in staining formalin fixed paraffin embedded tissue sections. Several antibodies against cell cycle regulated proteins are used as an operational marker for proliferation. One of the widely used antibodies is Ki-67 (Gerdes et al., 1983). Antibodies against PCNA are also useful reagents in assessing cell proliferation on paraffin embedded sections. We have collaborated with histopathologists to study the correlation between cell proliferation and PCNA staining using antibody PC10. This monoclonal antibody stained only the proliferating cells in various normal human and rat tissues. Except for breast cancers, other clinical materials, like lymphomas, gave a good correlation between PCNA index and S phase fraction. In breast cancer there appears to be no correlation between PCNA index and other indices of proliferation. The monoclonal antibody PC10 may therefore be used as reagent to assess the proliferating cells in formalin fixed paraffin embedded tissues.

Therefore, a set of anti-PCNA monoclonal antibodies have been raised which are capable of recognising different regions of the PCNA molecule. Five of the monoclonal antibodies recognise PCNA from fission yeast and insect cells. This suggest that their epitopes are directed against an evolutionarily conserved region. These antibodies would be useful in studying the structure and function of PCNA from various species. Since PCNA is a highly conserved protein these antibodies would be useful in identifying and cloning the cDNA of PCNA from other species. These monoclonal antibodies have been used to isolate the PCNA homologue in S. pombe and this is described in the next chapter.
Figure 4.1: Western blot of HeLa S100 with anti-PCNA monoclonal antibodies. The antibodies used were as follows: lane 1: PC1; lane 2: PC2; lane 3: PC3; lane 4: PC4; lane 5: PC5; lane 6: PC6; lane 7: PC7; lane 8: PC8; lane 9: PC9; lane 10: PC10; lane 11: PC11 and lane 12: PBS.
Figure 4.2: Immunoprecipitation of $^{35}$S methionine labelled CV-1 cells with anti-PCNA monoclonal antibodies. The antibodies used were lane 1: BG2; lane 2: PC1; lane 3: PC2; lane 4: PC3; lane 5: PC4; lane 6: PC5; lane 7: PC6; lane 8: PC7; lane 9: PC8; lane 10: PC9; lane 11: PC10 and lane 12: PC11. The PCNA associated proteins are marked.
Figure 4.3: SDS-PAGE analysis of the PCNA associated proteins. HeLa S100 was applied on an affinity column prepared with a mixture of five anti-PCNA monoclonal antibodies, PC1, PC2, PC3, PC8 and PC10. The PCNA associated proteins are marked.
Figure 4.4: Competitive ELISA of unlabelled anti-PCNA antibodies with monoclonal antibody PC3.
Figure 4.5: Competitive ELISA of unlabelled anti-PCNA antibodies with the labelled monoclonal antibody PC8
Figure 4.6: Competitive ELISA with unlabelled anti-PCNA antibodies with the labelled monoclonal antibody PC10.
Figure 4.7: Sandwich ELISA of PCNA using solid phase anti-PCNA antibodies and biotinylated antibody PC9.
Figure 4.8: Immunofluorescence staining of CV-1 cells with anti-PCNA monoclonal antibodies. (A) PC2, (B) PC9, and (C) PC4.
Figure 4.9: Immunofluorescence staining of tonsil and rectal carcinoma tissue section with monoclonal antibody PC10. Anti-PCNA monoclonal antibody PC10 was used to stain (A & B): tonsil sections and (C): rectal carcinoma sections. Bar A & C= 50 μM and B= 30 μM.
Figure 4.10: Western blot of insect cells with anti-PCNA monoclonal antibodies. The antibodies used were lane 1: PC1; lane 2: PC2; lane 3: PC4; lane 4: PC3; lane 5: PC10; lane 6: PC5; lane 7: PC8 and lane 8: PBS.
Figure 4.11: Western blot of *S. pombe* cells with anti-PCNA monoclonal antibodies. The antibodies used were lane 1: PC4; lane 2: PC2; lane 3: PC3; lane 4: PC10; lane 5: PC5; lane 6: PC8; lane 7: PBS. The molecular weight standards are shown on the left.
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Figure 4.12: Immunological reactivity of various fragments of PCNA with anti-PCNA monoclonal antibodies. + denotes a positive reaction on a western blotting, - denotes a negative reaction. BP3 is the incomplete *S. pombe* PCNA cDNA.
Figure 4.13: Epitope map of anti-PCNA antibodies. The map summarises the probable locations of the epitopes of the anti-PCNA antibodies mapped in this study and by others (Huff et al., 1990). RAPAb is rabbit polyclonal antibody raised against an N-terminus amino acid (11-23)synthetic peptide.
TABLE 4.1: Summary of the characteristics of the new anti-PCNA monoclonal antibodies

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</tr>
<tr>
<td>PC11</td>
<td>IgG1</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>+</td>
</tr>
</tbody>
</table>
### TABLE 4.2: Competitive inhibition between all the 11 anti-PCNA antibodies and biotinylated antibodies PC3, PC8, PC9 and PC10.

<table>
<thead>
<tr>
<th>Competing Antibody</th>
<th>C50* for biotinylated antibody</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PC3</td>
</tr>
<tr>
<td>PC1</td>
<td>0.4</td>
</tr>
<tr>
<td>PC2</td>
<td>0.3</td>
</tr>
<tr>
<td>PC3</td>
<td>0.8</td>
</tr>
<tr>
<td>PC4</td>
<td>-</td>
</tr>
<tr>
<td>PC5</td>
<td>4.0</td>
</tr>
<tr>
<td>PC6</td>
<td>0.8</td>
</tr>
<tr>
<td>PC7</td>
<td>-</td>
</tr>
<tr>
<td>PC8</td>
<td>0.3</td>
</tr>
<tr>
<td>PC9</td>
<td>-</td>
</tr>
<tr>
<td>PC10</td>
<td>0.1</td>
</tr>
<tr>
<td>PC11</td>
<td>0.4</td>
</tr>
<tr>
<td>BG2</td>
<td>-</td>
</tr>
</tbody>
</table>

* Microgram of competing antibody to give a 50% inhibition of labelled antibody binding.
† Too high to be significant.
CHAPTER 5

ISOLATION AND CHARACTERISATION OF PCNA GENE AND cDNA FROM FISSION YEAST

5.1: INTRODUCTION

The DNA replication machinery shows a high degree of structural and functional homology from mammals to yeasts. The functional homologues of polymerase α (Johnson et al., 1985) and polymerase δ (Bauer and Burgers, 1988a), thymidylate kinase (Sclafani and Fangman, 1984), thymidylate synthetase (Bisson and Thorner, 1977) and DNA ligase (Johnston and Nasmyth, 1978) had been identified in *S. cerevisiae*. Yeasts provide a good system for studying cell cycle regulated genes. They can be genetically manipulated to understand eukaryotic DNA replication. Foreign DNA can be transformed into the yeasts and its role can be studied by genetic complementation. Physically isolated genes can also be used to investigate their functions. The DNA can be mutated *in vitro* and reintroduced back to see the effect of the altered gene structure on its function.

The PCNA gene from *S. cerevisiae* has been cloned recently (Bauer and Burgers, 1990). It contains no introns. This gene is essential for the viability of budding yeast. The 26 kd *S. cerevisiae* PCNA can complement human PCNA in stimulating the activity of mammalian DNA polymerase δ (Bauer and Burgers, 1988a). When compared with the predicted amino acid sequence of human PCNA, this gene shows about 35 % homology. Although it is able to stimulate mammalian DNA polymerase δ activity, it is unable to substitute for human PCNA in SV40 DNA replication (Ng et al., 1990).

Five of the monoclonal antibodies, PC2, PC3, PC5, PC8 and PC10, raised against rat PCNA cross reacted with *S. pombe* PCNA. However, they failed to show any reaction with that of *S. cerevisiae*. This suggested that *S. pombe* PCNA might show closer homology with human PCNA. To this end we have isolated the PCNA gene and its cDNA from *S. pombe* and expressed it in *E. coli*. 

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5.2 METHODS AND MATERIALS

Screening of $\lambda$gt11 *S. pombe* cDNA library for PCNA cDNA with monoclonal antibody PC10

A *S. pombe* $\lambda$gt11 cDNA library was obtained from V. Simanis and screened according to the method of Young and Davis, 1983. To screen for a PCNA cDNA with the monoclonal antibody PC10 the $\lambda$gt11 *S. pombe* cDNA library was plated on a lawn of *E. coli* Y1090 and incubated at 42°C for 2 hrs. The expression of $\beta$-galactosidase fusion protein was induced by overlaying an IPTG saturated BA85 nitrocellulose membrane (Schleicher and Schuell, Germany) on the plaques followed by incubation at 37°C for overnight. The filters were removed from the plates and processed as for colony blotting (section 2.4). The positive plaques were isolated and replated and this process were repeated three times until all the plaques in a 90 mm petri dish showed positive reaction with the antibody.

Five PC10 positive independent plaques were obtained. Only one of the plaques reacted with all other four monoclonal antibodies (PC2, PC3, PC5 and PC8) and was used for further analysis.

Screening of *S. pombe* Laurist 4 cosmid library for PCNA gene

The *S. pombe* genomic Laurist 4 cosmid library was kindly provided by Dr. G. Zehetner (Imperial Cancer Research Fund, London). It was prepared by ligating an MboI partial digest of *S. pombe* genomic DNA into BamHI cloning site of cosmid Laurist 4 and plated onto the host ED8 767. The colonies were then replica plated on L agar plates overlayered with Hybond N+ membrane (Amersham). The $2 \times 10^4$ *S. pombe* Laurist 4 cosmid clones were screened with the PCNA cDNA obtained as above. Eighteen clone gave a positive signal with the PCNA cDNA obtained as above. Eighteen clone gave a positive signal with the PCNA cDNA. They were further analysed by southern blotting.

Primer extension analysis

The primer 5' ATCTGTAACCTACTCCTTGAT 3' corresponding to the position 167 on the PCNA gene (see Figure 5.2) was used for primer extension reaction. This primer was end-labelled with $^{32}$P at its 5' end. Using this labelled primer a reverse transcription reaction was performed on total RNA. For the reverse transcription reaction 10 $\mu$g RNA was taken and annealed to the labelled primer in 10 mM PIPES, pH 6.4, 400 mM KCl and 2 units/µl of RNAsin (Boehringer Mannheim, Germany). The reaction
was carried out at $42^\circ$C for 2 hrs in 222.2 mM Tris HCl, pH 8.3, 4.4 mM DTT, 6.66 mM MgCl$_2$, 1.11 mM of each dNTP, 0.11 units/$\mu$l of RNasin and 20 units of avian myoblastosis virus reverse transcriptase (Promega, USA). The product was treated with phenol/chloroform and ethanol precipitated. The pellet was dissolved in Stop Solution (95% formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol). This was then loaded on a 6% denaturing polyacrylamide gel.

**Primers used in the synthesis of PCNA cDNA**

Synthetic oligonucleotide primers were used for the amplification of the reverse transcribed RNA by PCR. The primers used for the amplification had a restriction site (underlined in the sequence shown below) incorporated to facilitate the cloning of the PCR amplified product. Primer 1 was used to reverse transcribe PCNA cDNA from the total RNA from *S. pombe*. The sequence of the primers used are:

1: 5' CTGTGGGATATGAT 3'
2: 5' TACGCATGTGCTGAGCT 3'
3: 5' TTGATTCGGATCCCTACTCCTCATCCT 3'

**Construction of plasmid pURA1016, ura4 and transformation in S. pombe**

For gene disruption analysis a construct was made in which a fragment of the PCNA gene was deleted and replaced with the orotidine-5'-phosphate decarboxylase (*ura4*) gene. For this the plasmid pIR2U containing 1.8 bp *ura4* gene was digested with *Hind*III and cloned into *Hind*III digested pBluescript. Plasmid DNA was purified from the colonies containing the cloned *ura4* gene. The *ura4* gene was removed from this construct by digesting with the restriction enzyme *Pst*I and *Xho*I and cloned into *Pst*I and *Xho*I digested pUR1016 to give pURA1016. This resulted in the removal of 330 bp from position 316 in the PCNA gene (see Figure 5.2).

The plasmid construct pURA1016.ura4 was digested with *Nde*I and *Xmn*I. This 3.4 kbp fragment containing deleted PCNA and *ura4* (*PCNA+::ura4*) was purified and transformed into $h+/h-$ *ura4*-D18/*ura4*-D18 ade6-M210/ade6-M216 leu1-32/leu1-32 diploid strain by the method of Moreno *et al.*, (1990). Stable transformants containing the integrated copy of the *in vitro* altered PCNA were selected by repeated plating on minimal media containing 75$\mu$g/ml leucine (Sigma Chemical Co. USA).
5.3: ISOLATION AND CHARACTERISATION OF PCNA GENE IN S. POMBE

Isolation of S. pombe PCNA cDNA from λgt11 library

The anti-PCNA monoclonal antibody PC10 reacted strongly with PCNA of S. pombe on a western blot as shown in chapter 4 and therefore it was used to screen a λgt11 library for PCNA cDNA. Out of the five positive plaques that showed positive reaction with PC10 only one reacted very strongly with all the other four monoclonal antibodies, PC2, PC3, PC5 and PC8, that cross reacted with S. pombe PCNA. The insert from this plaque was subcloned in pBluescript (Strategene, USA). The 800 bp insert was sequenced from end to end. The predicted open reading frame of this insert was compared with the predicted amino acid sequence of human and rat PCNA. The insert had 83 amino acid missing at its amino terminus. This was further confirmed by comparing the sequence of this clone with the PCNA gene isolated later.

Isolation of S. pombe gene from Laurist 4 cosmid library

To determine the structure of PCNA gene a genomic library was screened using the incomplete cDNA as a probe. Eighteen clones showed positive reaction with the PCNA cDNA clone. To further confirm that the reaction was genuine the cosmid DNA was purified from these colonies and analysed by restriction digestion with EcoRI and EcoRV along with the S. pombe genomic DNA. Only three clones, ICRFc60C1233, ICRFc60D1016 and ICRFc60C0210, showed the same digestion pattern as that of the genomic DNA. The southern blot obtained with ICRFc60D1016 and genomic DNA is shown in Figure 5.1. With EcoRI only one fragment of 2.1 kb was obtained whereas with EcoRV two fragments of 5.5 and 1.8 kb were obtained. Since EcoRI gave only one band on a Southern blot that was identical with the size obtained with the genomic DNA, the EcoRI fragment of ICRFc60D1016 was subcloned in pUR292 and sequenced completely. This plasmid construct was designated as pUR1016.

Structure of PCNA gene

The 2.1 kb PCNA gene was sequenced in both directions using 21mer oligonucleotide primers. The sequence is shown in Figure 5.2. The sequence of this gene was compared with the sequence of other known PCNA genes. An intron of 108 bp was identified at position 173 by this sequence comparison. The predicted intron contained three translational termination
codons in the predicted open reading frame. There was a palindromic sequence with 8As and 8Ts in the intron. The splice junctions were determined by sequencing the PCR-amplified cDNA prepared from the total RNA using *S. pombe* PCNA specific primer. The splice donor and acceptor sites are indicated in Figure 5.2 and are in good agreement with the consensus splice donor and acceptor site reported for *S. pombe* (Kaufer et al., 1985). The splice site consensus CTNAC was also found at 3' end of the intron marked in the figure 5.2. By comparing the predicted amino acid sequence of other known PCNA the translational start site was determined. The 2.1 kb PCNA gene had 843 bp at the 5' end from the ATG and 497 bp at the 3' end after the translational termination codon. The position of the poly A addition as found in the cDNA sequence was at position 1163.

The transcriptional start site of the PCNA gene was mapped by primer extension from total RNA (Figure 5.3). The transcriptional start site was located 92 bp upstream of the first initiation codon ATG and is indicated by +1 in figure 5.2. In *S. pombe* the TATA box is typically located 25-30 bp upstream of the transcriptional start site (Russell, 1983). However, in *S. pombe* PCNA gene there are two sequences similar to TATA box: one is located at position -114 and the second at -85. Various motifs for DNA binding proteins found in the 5' untranslated region are shown in Figure 5.4. A perfect octamer motif ATGCAAAT (Chellappan and Nevins, 1990) is present at position -238, an upstream control element (UCE-1) (Schnapp et al., 1990) is present at -166. A CCAATT box is located at -574 and an AP-1 site (Curran and Franzia, 1988) at -375. In the AT rich 5' region, the sequence AAAATATAA is repeated four times at -131, -140, -524 and -567.

5.4: CHARACTERISATION OF PCNA cDNA FROM *S. POMBE*

From the information obtained from the PCNA gene a complete cDNA of PCNA was synthesized. This cDNA was then further characterised.

**Synthesis of complete PCNA cDNA**

As mentioned previously the cDNA of PCNA obtained by screening the λgt11 cDNA library lacked 83 amino acid at its N terminus. The PCNA gene isolated from Laurist 4 genomic library using this cDNA seems to be complete. By amino acid sequence comparison with other PCNA genes suggest that the translation should start at position 92 of the *S. pombe*
PCNA gene. In fact there was an ATG 92 nucleotide downstream of the transcriptional start site.

To get a complete PCNA cDNA the total RNA from *S. pombe* was reverse transcribed using PCNA specific primer 1 (for sequence see to section 5.2). The reverse transcribed product was amplified by PCR using primer 2 and 3. The primer 2 had an *Nde*I site and primer 3 had a *BamHI* site. The amplified product after digestion with *Nde*I and *BamHI* were cloned and expressed in pT7.7 vector. The cloned PCNA cDNA was confirmed by sequencing at both ends.

**Characterisation of PCNA cDNA**

The PCNA cDNA from *S. pombe* was expressed in pT7.7. The expressed protein was recognised by the monoclonal antibodies PC2, PC3, PC5, PC8 and PC10. These antibodies recognised two protein bands of apparent molecular weight of 36 kd and 30 kd on a western blot. This pattern was obtained in all the colonies expressing *S. pombe* PCNA. The mobility of the 36 kd protein band was identical to that of PCNA present in *S. pombe* total cell extract (Figure 5.5). The 30 kd protein band might be a proteolytic degradation product of the 36 kd protein band. Another possibility may be the presence of another translation start site near the actual site. Indeed there are two other methionine present at position 41 and 51, beside at position 1, in the *S. pombe* cDNA. The methionine at position 41 is conserved in human, rat, *Drosophila*, *Xenopus* and *S. pombe* whereas the other methionine is unique to *S. pombe* (Figure 5.6). Since a single protein band was obtained when rat PCNA cDNA was cloned into the same restriction sites, it seems likely that the 30 kd band is the product of the protein starting at methionine 51.

**5.5: PCNA GENE DISRUPTION**

A linear 3.4 kbp *PCNA+::ura4+* fragment was transformed into a diploid strain of *S. pombe*, which lacked an endogenous *ura4* gene, to disrupt one of the copies of the PCNA gene in this strain. The cells containing the altered gene could be selected by their ability to grow in the absence of uracil. Following this procedure 24 colonies were obtained after transformation with the *PCNA+::ura4+* fragment. To obtain stable transformants produced by integration of this fragment in the *S. pombe* genome these *ura4+* colonies were allowed to grow without selection and then the selection was applied. This process was repeated three times. Only
two stable *ura4+* stable integrants were obtained after the third plating and were named as 10ΔPCNA and 17ΔPCNA.

The chromosomal DNA was purified from these two mutants. To confirm that the integration had occurred at the PCNA locus they were analysed by southern blotting. Since the sequences of the PCNA and *ura4* gene were known the sizes of the fragment obtained by different restriction enzymes could be worked out. The restriction enzymes used were *EcoRI*, *EcoRV* and *HindIII*. The southern blot was probed with (a) complete *EcoRI* fragment of the PCNA gene, (b) *HindIII* fragment containing *ura4* gene and (c) the 330 bp *PstI* and *XhoI* fragment of the PCNA gene which was replaced with *ura4* gene. A Southern blot probed with the *EcoRI* fragment of the PCNA gene is shown in Figure 5.7.

Analysis of Southern blot showed that the integration of the altered PCNA gene occurred at the PCNA locus in 17ΔPCNA but not in 10ΔPCNA. Tetrad analysis of these two mutants was then performed. Four tetrads were dissected for each of these mutants. In 17ΔPCNA out of the 16 tetrad dissected 4 were viable and these were *ura4-* showing that the PCNA was essential for the viability of the spore. In 10ΔPCNA all the 16 spores were viable and 4 of these were *ura4-*-.

The 17ΔPCNA diploid strain was sporulated and the spores were germinated in the absence of uracil to select for *ura+* haploid cells. These germinated spores were then examined for the presence of any phenotype. Eighty percent of this germinated strain arrested as elongated cells (Figure 5.8). Similar results were obtained when the non-viable tetrads from 17ΔPCNA were examined. About 20% cells were apparently of normal size.

5.6: CELLULAR LOCALISATION OF *S. POMBE* PCNA

Five monoclonal antibodies, PC2, PC3, PC5, PC8 and PC10, recognised PCNA from total cell extract of *S. pombe* on a western blot. Only PC8 and PC10 was able to localise PCNA in the cells by immunofluorescence. PCNA in *S. pombe* as in the mammalian cells was immunolocalised in the nuclei (Figure 5.9).

5.7: DISCUSSION

Monoclonal antibodies to rat PCNA was used to isolate its cDNA from *S. pombe*. This was then used to isolate the complete PCNA gene along with the untranslated regions at both ends. The sequence information from
the gene was then used to synthesize a complete PCNA cDNA. This cDNA when expressed in *E. coli* was recognised by the five anti-PCNA monoclonal antibodies that also recognised this protein in total cell extract of *S. pombe*.

The open reading frame of PCNA cDNA in *S. pombe* encodes 261 amino acid with predicted molecular weight of 28,600. Comparison of this amino acid sequence with the predicted amino acid sequence of other known PCNA proteins revealed several features. The helix-turn-helix motif, a putative DNA binding domain, at amino acid 61 to 77 is conserved in *S. pombe* PCNA with few preferred amino acid substitutions. The leucine repeats at the C terminus are also conserved. The amino acid sequence of *S. pombe* PCNA was compared with PCNA from human (Almendral et al., 1987), *S. cerevisiae* (Bauer and Burgers, 1990), *Drosophila* (Yamaguchi et al., 1990) and *Xenopus* (Leibovici et al., 1990)(Figure 5.6). *S. pombe* PCNA shows 50% amino acid identity with human PCNA but only 23% with *S. cerevisiae* PCNA. The C terminus of the protein shows a high degree of homology with human, rat, *Drosophila* and *Xenopus* PCNA. Thus 25 amino acid residues between 216 and 240 are identical in *S. pombe*, human, rat, *Drosophila* and *Xenopus* PCNA whereas only 4 of these are present in *S. cerevisiae*. Interestingly, PCNA from *S. cerevisiae* does not complement human PCNA in SV40 DNA replication whereas *Drosophila* PCNA does. This suggests that the C terminus might have a functional role in SV40 DNA replication. If this is the case then PCNA from *S. pombe* might complement the human PCNA in DNA replication.

The PCNA gene is essential for the viability of *S. pombe*. A null mutant of PCNA was created by deletion of a 330 bp fragment and replacement with 1.8 kbp of *ura4* gene. Null mutants arrested as elongated cells. However a small number of cells showed apparently normal size. This suggests that a couple of division occurred normally before the cells were arrested. It seems likely that the germinated cells utilised maternal PCNA for a first few cycles before needing to synthesize their own. Similar results had been reported in *Drosophila* (Yamaguchi et al., 1990) and *Xenopus* (Leibovici et al., 1990) where PCNA is stockpiled in unfertilized eggs and this was utilised later during various developmental stages.

In human the PCNA gene is transcriptionally and post-transcriptionally regulated (Chang et al., 1990). The level of PCNA mRNA is growth regulated, e.g., in quiescent cells no PCNA mRNA is detected however, when quiescent cells are stimulated with foetal calf serum the
PCNA mRNA level is increased within 12 hrs and plateau in 16-18 hrs. In growth dependent regulation of PCNA intron 4 in human PCNA gene has been implicated (Ottavio et al., 1990b). Thus the presence of single intron in *Drosophila* and *S. pombe* (although not at the same position) may play an important role in the regulation of PCNA.

Various DNA binding motifs were found at the 5' end of *S. pombe* PCNA gene. In addition to the TATA box and CCAATT box, an octamer motif was also found at the 5' end of the PCNA gene. Although the octamer binding proteins are not yet identified in *S. pombe*, these motifs are critical in the cell cycle specific transcription of H2b gene in mammalian cells (LaBella et al., 1988). The octamer binding proteins are also the target of several viral transcriptional factors notably VP16 of the herpes viruses (O'Hare and Goding, 1988). It is conceivable that this motif is involved in the regulation of PCNA.

The anti-PCNA monoclonal antibodies, PC8 and PC10, immunolocalised PCNA in the nucleus of fission yeast. The level of PCNA does not seem to vary significantly throughout the cell cycle. These monoclonal antibodies could be used to immunolocalise PCNA in various cdc mutants. It is possible that mutations in certain genes would influence the expression of PCNA. This will be important in unravelling the connections between cell cycle control genes and the DNA replication apparatus.
Figure 5.1: Southern blot of *S. pombe* genomic DNA and cosmid ICRFc60D1016. *S. pombe* genomic DNA (lane 1,2) and cosmid ICRFc60D1016 (lane 3,4) were digested with *Eco*RV (lane 1,3) and *Eco*RI (lane 2,4). and probed with $^{32}$P labelled incomplete *S. pombe* PCNA cDNA probe.
Figure 5.2: Complete nucleotide sequence and deduced amino acid sequence of *S.pombe* PCNA gene. The transcriptional start site is marked by +1. The position of poly A tail is marked on the 3' end of the sequence. The consensus splice junctions of the intron are underlined.
Figure 5.3: Primer extension analysis of *S.pombe* PCNA gene. $^{32}$P labelled 21 mer oligonucleotide was used for the mapping of the transcriptional start site. The same primer was used for sequencing the plasmid construct pUR1016 and run alongside the reverse transcribed product. Presence and absence of reverse transcriptase is shown by + and -, respectively. The reverse transcribed product is shown by an arrow.
Figure 5.4: Nucleotide sequence at the 5' end of *S. pombe* PCNA gene. The consensus sequence for the binding of various transcription factors found at the 5' untranslated sequence are underlined. The nucleotide position are numbered on the right relative to the transcriptional start site.
Figure 5.5: Western blot of *S. pombe* PCNA cDNA expressed in pT7.7 with monoclonal antibody PC10. Lane 1: total cell extract of *S. pombe*; total cell extract of pLysS BL21(DE3) containing lane 2: pT7.7 and lane 3: pT7PCSP.
Figure 5.6: Comparison of deduced amino acid sequence of *S. cerevisiae* (cer), *S. pombe* (pom), human (hum), *Drosophila* (dro) and *Xenopus* (xen) PCNA. The amino acid identical between human PCNA and the rest are marked in bold.
Figure 5.7: Southern blot analysis of the diploid mutant strains of *S. pombe*. Diploid *S. pombe* cells containing an integrated copy of the PCNA::*ura4* was probed with EcoRI fragment of *S. pombe* PCNA gene. Lane 1, 4, 7 are the genomic digest of the control DNA from diploid strain of *S. pombe*; lane 2, 5, 8 are that of 17ΔPCNA and lane 3, 6, 9 are 10ΔPCNA. Lane 1, 2, 3 contain DNA digested with EcoRI; Lane 4, 5, 6 contain DNA digested with EcoRV and Lane 7, 8, 9 contain DNA digested with HindIII.
Figure 5.8: Cell morphology of the null mutants of PCNA in *S. pombe*. Panel A shows the cells stained with DAPI and panel B is the phase contrast of the same field. Bar=10μM.
Figure 5.9: Immunofluorescence staining of *S.pombe* cells. Panel A shows the *S.pombe* cell stained with PC8 and panel B is the phase contrast of the same field.
CHAPTER 6

DISCUSSION

In this chapter the results obtained in the previous chapters are summarised and also the directions in which this work will be extended in future are described.

Bacterial over-expression and purification of PCNA

Purification of PCNA from mammalian source can be achieved by fractionation of the soluble cytoplasmic extract from various tissue cultured cell lines (e.g., HeLa, 293 cells) (Prelich et al., 1987) or from tissues (e.g., thymus) (Ogata et al., 1987b). Biochemical purification of PCNA required at least four chromatographic steps. The S100 fraction of the cellular homogenate are taken through two ion exchange column, phosphocellulose and DEAE cellulose. The bound material after DEAE column is loaded on a phenyl Sepharose followed by Q-Sepharose column. The protein is finally fractionated on a glycerol gradient. Following this procedure the yield of PCNA from 32 litres of a human cell line 293 (total protein about 1 gm) was 48 µg (Prelich et al., 1987). To obtain a large amount of soluble PCNA bacterial expression was a good and easy alternative. We have used pRIT2T (Nilson et al., 1985) and pT7.7 (Tabor and Richardson, 1985) for the expression of PCNA. Expression of PCNA in pRIT2T had two advantages, first, the protein was soluble and the fusion protein had protein A attached at its N terminus so the purification was essentially a one step affinity chromatography on IgG Sepharose. Secondly, since the protein is not exposed to strong chaotropic reagents and protein A itself is a small molecule (molecular weight of 27 kd) there is a greater chance that the protein will retain its native structure and therefore can also be used in various functional assays where the protein A moiety can be used as a tag to localise PCNA.

The second vector pT7.7 used in this study utilises the T7 polymerase system and therefore the expression of even toxic proteins can be carefully controlled. The protein obtained by pT7.7 vector can be expressed as an unfused protein. There is an Ndel site at the first initiation
codon into which the sequence to be subcloned can be manipulated such that 
the translation starts at the first initiation codon of the cloned sequence. 
Rat PCNA cDNA expressed in this way was found to be in the soluble 
fraction of the cell extract. Recently it was shown that bacterially 
expressed human PCNA was functionally active in \textit{in vitro} SV40 DNA 
replication assays (Tsurimoto and Stillman, 1990). Thus a source of 
unfused PCNA had been established which could be useful for the functional 
and biochemical characterisation of PCNA.

Mammalian PCNA is a homodimer (Prelich \textit{et al.}, 1987). It is not 
yet known whether monomer or dimer of PCNA is the active component in 
DNA replication. Since PCNA expressed in pT7.7 was able to dimerise in 
solution conditions affecting the association of the PCNA monomer can be 
studied. Mutant PCNA can be generated by site directed mutagenesis such 
that they are unable to form dimers. These mutants can be used to show 
that dimer or monomers are essential for DNA replication. PCNA cDNA 
from different species expressed similarly can be mixed to see whether 
they can exchange subunits to form heterodimer and whether these are 
replication competent.

Protein A-PCNA fusion protein could be used to study the functional 
properties of PCNA. The effect of over-expression of PCNA in a cell has 
not yet been studied. This can be done either by transfection or by 
microinjection. In these studies a specific probe is required to differentiate 
the endogenous PCNA from the exogenous PCNA. Protein A-PCNA fusion 
protein could be microinjected and the fate of the microinjected protein 
could be traced by detecting protein A using immunoglobulin.

**Monoclonal antibodies to PCNA**

Using the bacterially expressed PCNA as an antigen, eleven anti-
PCNA monoclonal antibodies were raised. These monoclonal antibodies 
recognised PCNA in total cell extract of HeLa on a western blot. Except for 
PC11 all the other monoclonal antibodies were able to recognise and 
immunoprecipitate PCNA from total cell extract. From the results of 
competition ELISA and epitope mapping ten of the monoclonal antibodies 
recognise the central region of PCNA. PC7 showed very weak reactivity on 
a western blot but was able to recognise PCNA expressed by the plasmid 
construct pC10 by ELISA.

Eleven monoclonal antibodies raised against rat PCNA are a set of 
immunological reagent that could be used to understand the role of PCNA.
Two of the monoclonal antibodies, PC2 and PC10, beside showing strong reaction with PCNA on a western blot also show weak reactivity with an additional protein band of 120 kd. Similar cross reaction had been reported for the two monoclonal antibodies, 19A2 and 19F4 (Ogata et al., 1987b), and some autoimmune sera specific for PCNA (Chan et al., 1985). There seems to be another protein in mammalian cells having some homology with central region of PCNA. It would be interesting to identify this protein and see the basis of cross reaction. These cross reaction are not uncommon with monoclonal antibodies. One of the monoclonal antibodies against SV40 large T antigen recognised another protein of 68 kd (Lane and Hoeffler, 1980). This protein, called p68, was latter identified as an RNA dependent helicase (Ford et al., 1988, Iggo and Lane, 1989).

PCNA associated proteins

In vitro SV40 DNA replication system can be reconstituted using seven biochemically purified proteins, namely DNA polymerase α-primase complex, topoisomerase I and II, RF-C, PCNA, PP2AC and DNA polymerase δ (Weinberg et al., 1990). These proteins were purified by fractionating the soluble extract. During this purification procedure RNAse H and DNA ligase activity is lost. Addition of RNAse H, DNA ligase I and 5' exonuclease (Goulian et al., 1990, Ishimi et al., 1988) purified by an alternative procedure together with the seven other known proteins generated closed circular replicated SV40 DNA.

DNA replication requires an interplay of several proteins. PCNA had been shown to be involved in the leading strand synthesis and also for the coordination of the leading and lagging strand synthesis (Prelich and Stillman, 1988). PCNA must be interacting with other replication associated proteins. Studying this protein-protein interactions would lead us to understand the different interactions that PCNA is involved in different stages of DNA replication. One of the ways to study these interactions is by analysing the immunoprecipitates containing PCNA. Careful analysis of the immunoprecipitate showed that there were at least four proteins of 45 kd, 25 kd, 20 kd and 18 kd, associated with PCNA. The identity of these proteins are not known. The small polypeptides being the proteolytic fragments of PCNA cannot be ruled out. On the other hand, it is possible that these polypeptides might be one of the subunits of a large protein. The apparent molecular weights of these proteins do not correspond to any of the known proteins, pol δ (170 kd, 48 kd) (Syvaoja et
al., 1990), RFC (140, 41, 37 kd) (Tsurimoto and Stillman, 1990) and pol α (140 kd) (Lehman and Kaguni, 1989) which had been implicated in interactions with PCNA. The 45 kd polypeptide might be either one of the subunits of pol δ (48 kd) or that of RFC (41 kd). However, the possibility that this protein is actin (45 kd) cannot be ruled out. It is also possible that the 25 kd and 18 kd proteins are associated with actin and not associated with PCNA at all. These questions have to be investigated further before looking for the activity associated with these proteins.

**Epitope mapping**

Several significant features became apparent by epitope mapping of the monoclonal antibodies to PCNA. The monoclonal antibodies were raised against soluble protein A-PCNA fusion protein which was hoped to retain overall native conformation. However, when the epitope of the antibodies were mapped they were localised within the central region of the molecule. The epitope of the monoclonal antibodies, 19A2 and 19F4, raised previously against the denatured PCNA also localised within this region (Huff et al., 1990). The epitopes of 14 autoimmune sera against PCNA had also been mapped (Huff et al., 1990). Although these antisera showed very different reactivities with PCNA, nearly all mapped between 60-150 amino acids. Some of these antisera also required the C terminus to show their reactivity with PCNA. No antibody has yet been found which recognises the N or C terminus of PCNA when full length protein was used as an antigen. Data from the epitope mapping also showed that the antibodies raised against PCNA in SLE patients recognise conformation dependent epitope. Most of these autoimmune antisera neither react with the 15 mer synthetic peptide nor the fusion protein on a western blot. On the other hand the antibodies raised experimentally recognised the synthetic peptide as well as β-galactosidase-PCNA fusion protein (Huff et al., 1990). Some of the autoimmune sera had been able to neutralise the activity of PCNA in a replication assay (Tan et al., 1987). Why are the antibodies all against the central region of the molecule? One possibility is that this region is exposed whether the antigen is native or denatured and thus strongly antigenic. In homodimer of PCNA the central region might be on the outer surface of the dimer whereas the other sites are either hidden within the dimer or form a part of the compact structure of monomer. Since PCNA in the SLE patients might be present as a dimer, which may be a part of a large protein complex, the antibodies were mostly conformation dependent.
and against the exposed central region. PCNA used as an antigen in experimentally induced antibody, as in this study, although forming a dimer was presumably not associated with any other protein.

To study the structure function relationship of PCNA, antibodies against various region of the molecule should be produced. The affect of these antibodies can then be studied \textit{in vitro} in replication assays and also \textit{in vivo} by microinjection and thus would be useful in analysing the functional role of various regions of PCNA. Furthermore, it would also enable us to understand the conformation alterations of PCNA within the cell cycle. One monoclonal antibody PC9 which recognised an epitope near the C terminus of the PCNA shows markedly different pattern on CV1 cells. It only stains the nucleolus in these tissue culture cells. It would be interesting to analyse the immunostaining pattern obtained with antibodies raised against other regions of the molecule.

\textbf{Tissue staining}

Monoclonal antibodies PC2, PC5, PC6, PC8, PC10 and PC11 stained the nuclei of proliferating cells in formalin fixed paraffin embedded tissue sections. Among these PC10 showed the strongest staining. We have collaborated with histopathologists at Guy's Hospital, London, to study the correlation between the proliferating index and PC10 staining on tissue sections (Hall \textit{et al.}, 1990). Several clinical samples showed good correlation between proliferation index and PCNA. Some of these samples showed aberrant expression of PCNA in the apparently normal surrounding tissues of the carcinoma. It seems likely that the production of some growth factors induced the expression of PCNA in the surrounding tissues. The PCNA gene is regulated by growth factors like PDGF (Jaskulski \textit{et al.}, 1988b). The PCNA promoter is also responsive to E1A (Jelsma \textit{et al.}, 1989). It would be interesting to identify this growth factor and see its effect on the expression of PCNA \textit{in vitro}. If PCNA is responsive to this growth factor then the antibodies can be used to rapidly identify these growth factors in the tumour samples.

In tissues from rat thymus there was a good correlation between the cells in the S phase, bromodeoxyuridine incorporation and PCNA staining when monoclonal antibody PC10 was used. However HeLa cells showed no correlation between PC10 staining and the proliferation index. This is consistent with the fact that HeLa are rapidly proliferating cells. Similar results have been reported previously using synchronised culture
of HeLa cells (Morris and Mathews, 1989). Thus PC10 antibody could be useful in the detection of the rapidly proliferating cells in the presence of apparently normal quiescent cells.

The monoclonal antibody 19A2 had been used previously to study the correlation between PCNA staining and the proliferation index (Garcia et al., 1989). This monoclonal antibody 19A2 recognise PCNA only when the tissue is fixed with methacran. It fail to recognise PCNA in either formalin fixed or frozen sections. Monoclonal antibody PC10 recognise PCNA in formalin fixed paraffin embedded tissue sections and also works well in methacran fixed samples. Thus anti-PCNA monoclonal antibody, PC10 can be used as a proliferation marker even in archival material.

Yeast PCNA in replication

The gene of PCNA in *S.pombe* was isolated from a laurist 4 cosmid library. It was cloned and sequenced. The 2.1 kb PCNA gene in *S. pombe* contains a single intron at nucleotide 173. The cDNA was synthesized from the sequence information obtained from the gene. The predicted open reading frame encodes for a 261 amino acid protein. Amino acid sequence comparison of this PCNA with that of other species revealed high degree of amino acid identity. *S. pombe* PCNA showed 55% amino acid identity with human PCNA and if the conserved amino acid residue are taken into account then the homology increases to 70%. The amino acid identity was greater at the C terminus than at the N terminus.

Cloning of *S. pombe* PCNA gene opens up a new approach to understanding the role of PCNA. The cell cycle of fission yeast is relatively well understood. It also provides a good system for genetic manipulations. Cloning of the PCNA gene from *S. pombe* will allow us to study its genetics. We, for example, study the consequences of overexpressing PCNA on cell growth and division. The PCNA gene can also be used for generating temperature sensitive mutants which would be useful in analysing the function(s) of PCNA. Furthermore, several well characterised cdc mutants are available. Double cdc mutants can be generated and the steps where PCNA is required can be identified. Some of the conditional cdc mutants available have not been characterised. Transformation with PCNA cDNA would tell us whether any of them are for PCNA.

The promoter of *S. cerevisiae* PCNA gene shows a characteristic 14 bp stretch of sequence identical to that of DNA polymerase III in yeast (Bauer and Burgers, 1990). They are expressed identically throughout the
cell cycle which might suggest that this sequence might be involved in the regulation of the PCNA and DNA polymerase III genes. However no such element is found in the upstream sequence of the *S. pombe* PCNA gene. A systematic analysis of the 5' untranslated region of the PCNA gene would allow us to map the promoter region. It would be interesting to see how the PCNA gene is regulated in *S. pombe*. This can also be studied using various cdc mutants.

PCNA had been shown to be essential for the SV40 DNA replication. PCNA from *Drosophila* complemented human PCNA in *in vitro* SV40 DNA replication assay (Ng *et al.*, 1990) suggesting that this replicative function is conserved from mammals to insects. Although *S. cerevisiae* PCNA was able to stimulate mammalian pol δ it was unable to complement human PCNA in the SV40 DNA replication assay (Ng *et al.*, 1990). It would be interesting to see whether *S. pombe* PCNA can complement human PCNA in SV40 replication. Also hybrid molecule formed between PCNA from different species will permit the localisation of the region required for SV40 DNA replication.
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APPENDIX


Monoclonal antibody analysis of the proliferating cell nuclear antigen (PCNA)

Structural conservation and the detection of a nucleolar form

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Summary

The proliferating cell nuclear antigen, PCNA, has recently been identified as the polymerase δ accessory protein. PCNA is essential for cellular DNA synthesis and is also required for the in vitro replication of simian virus 40 (SV40) DNA where it acts to coordinate leading and lagging strand synthesis at the replication fork. The cDNA for rat PCNA was cloned into a series of bacterial expression vectors and the resulting protein used to immunize mice. Eleven new monoclonal antibodies to PCNA have been isolated and characterized. Some of the antibodies recognize epitopes conserved from man to fission yeast.

Immunocytochemical analysis of primate epithelial cell lines showed that the antibodies recognized antigenically distinct forms of PCNA and that these forms were localized to different compartments of the nucleus. One antibody reacted exclusively with PCNA in the nucleolus. These results suggest that the PCNA protein may fulfil several separate roles in the cell nucleus associated with changes in its antigenic structure.

Key words: proliferating cell nuclear antigen, monoclonal antibodies, nucleolus, nucleoplasm, yeast PCNA, insect PCNA.

Introduction

The proliferating cell nuclear antigen (PCNA) (Takasaki et al. 1984), also known as cyclin (Bravo and Celis, 1980; Bravo et al. 1981; Mathews et al. 1984) or as auxiliary protein for polymerase δ (Tan et al. 1986; Prelich et al. 1987b; Bravo et al. 1987), is required for simian virus 40 (SV40) DNA replication in vitro (Prelich et al. 1987a). Experiments using anti-sense oligonucleotides and micro-injection of antibodies strongly suggest that PCNA is also essential for cellular DNA synthesis (Jaskulski et al. 1988; Zuber et al. 1989a). It is required for leading strand synthesis in the SV40 system where it probably acts as an auxiliary protein for polymerase δ, coordinating leading and lagging strand synthesis and rendering the polymerase more processive (Prelch and Stillman, 1988). PCNA appears to be a cell cycle-regulated protein when examined by immunofluorescence (Celis and Celis, 1985). When cells are fixed using organic solvents PCNA staining is localized at the intranuclear sites where DNA synthesis is taking place (Bravo and Macdonald-Bravo, 1987). The predominant distribution of PCNA appears to change with the stage of the cell cycle. In early S phase PCNA has a very granular distribution and is absent from the nucleoli, while at late times in S phase prominent staining of the nucleoli is evident. In cells fixed using aldehydes, however, the distribution of PCNA is different and intense diffuse nuclear staining is evident throughout the cell cycle (Bravo and Macdonald-Bravo, 1987). This difference has been explained by the proposal that there are two forms of PCNA: a soluble form lost on organic solvent fixation and not involved in replication, and an insoluble form associated with the sites of on-going DNA synthesis (Bravo and Macdonald-Bravo, 1987). Consistent with this hypothesis the total concentration of PCNA varies at the most by only two- to threefold during the cell cycle but there is a greater fraction of PCNA that is insoluble due to chromatin association in S phase than in other phases of the cell cycle (Morris and Mathews, 1989). The concentration of PCNA present in the cell nucleus seems to be in excess of that required for its replicative function (Morris and Mathews, 1989).

PCNA is a very conserved protein as can be seen by the amino acid sequence homology between mammalian PCNAs (Mouriuchi et al. 1986; Matsumato et al. 1987; Almendral et al. 1987; Ogata et al. 1985). The gene for PCNA is present not only in mammals (Matsumato et al. 1987; Almendral et al. 1987) but also in plant cells (Suzuka et al. 1989). Recently a gene closely homologous to PCNA was found in the genome of baculovirus Autographa californica (O'Reilly et al. 1989).

Three per cent of the patients suffering from systemic lupus erythematosus, an autoimmune disease, have antibodies against PCNA (Miyachi et al. 1978) and it was through the use of sera from these patients that the protein was first defined. These polyclonal antibodies have been used for in vitro and in vivo studies of PCNA (Wong et al. 1987; Tan et al. 1987). An autoantibody against PCNA, AK, capable of immunoprecipitating PCNA from cell extracts had been shown to inhibit DNA polymerase δ activity (Tan et al. 1987; Zuber et al. 1989a). It recognizes epitopes localized at the N terminus of PCNA (Ogata et al. 1989)
Two monoclonal antibodies designated 19A2 and 19F4 have been raised against rabbit PCNA. By Western blotting they recognize PCNA from different mammalian species (Ogata et al. 1987a) and from Xenopus (Zuber et al. 1989b). However, they are unable to recognize PCNA from Saccharomyces cerevisiae (Bauer and Burgers, 1988). They are also unable to immunoprecipitate PCNA from any source (Tan et al. 1987) and they do not inhibit plasmid replication (Zuber et al. 1989a). Their epitopes have been localized to the central region of the PCNA molecule (Ogata et al. 1987b).

Here we report the expression of recombinant PCNA genes in Escherichia coli, and the production and characterization of 11 new monoclonal antibodies, which define localized to the central region of the PCNA molecule replication (Zuber et al. 1987). These antibodies were able to immunoprecipitate the source (Tan et al. 1987) and they are unable to recognize PCNA from any source (Zuber et al. 1989a). Their epitopes have been localized to the central region of the PCNA molecule (Ogata et al. 1987b).

Materials and methods
Preparation of cDNA constructs
The cDNA of rat PCNA, PCR-1 in pBR322 was obtained from Morishita (Matsuzato et al. 1987). It was subcloned into the β-galactosidase expression vector pUR288 (Ruther and Muller-Hill, 1983). In order to do this, pUR288 was partially digested with EcoRI, filled with Klenow fragment and digested again with XbaI. An XbaI and HincII digestion of PCR-1 gave two fragments of 1.0 and 1.1 kb (1 kb = 10 base-pairs). The 1.0 kb fragment containing the PCNA cDNA was purified and ligated into XbaI and the blunt-ended pUR288 vector to give pC288.

A second construct was made in the protein A expression vector pHIT2T obtained from Pharmacia (Nilson et al. 1987). A 728 bp (base-pair) fragment containing part of the PCNA cDNA in pC288, obtained by XbaI and PstI digestion, was subcloned into pHIT2T to give pC27T. This construct lacked the 3'-end 93 nucleotides of PCNA. E. coli N4830-1 cells, which contain a heat-labile 3-1-kDa enzyme, were capable of recognizing the fusion protein obtained from pC27T. The final boost was carried out on two successive days: one intraperitoneally and the other intravenously. Three days after the final intravenous boost the boost was killed and the spleen removed. The spleen cells were fused with mouse myeloma cells Sp2/O-Ag14 using 50% polyethylene glycol 1500 (Harlow and Lane, 1989). The cells were grown in Dulbecco's modified Eagle's medium (MEM) containing 20% foetal calf serum (FCS) and supplemented with interleukin 6 at 4 ng ml⁻¹ as growth factor (E. Weiss, personal communication). Hoxpoxanthine and azaserine were used to specify the hybridoma cells. The fusion was screened by cell staining of CV-1 cells (immortalized line of monkey kidney epithelial cells) and Western blotting of HeLa S-100 fraction (see below).

Purification of the fusion proteins
Protein A fusion protein. A bacterial culture of E. coli N4830-1 cells containing pC27T was grown and induced according to the manufacturer's instructions. The cells were pelleted at 7000 revs min⁻¹ in a GSA 60 rotor in a Sorvall RC-5B centrifuge for 15 min. The pellet was suspended in 10 mM sodium phosphate buffer containing 150 mM sodium chloride, pH 7.4 (PBS), and treated with 1 mg ml⁻¹ lysozyme. After sonication for 20 s at maximum amplitude, it was spun at 30000 revs min⁻¹ in a 40T rotor in a Beckman L-85M centrifuge for 30 min. The supernatant was applied to a column of human IgG-Sepharose 6 Fast Flow (Pharmacia). The column was washed with 50 vols of PBS containing 1 M sodium chloride and 0.1% Nonidet P40 (NP40). The bound protein A–PCNA fusion protein was eluted with 100 mM glycine–HCl, pH 3.0. The fractions were immediately neutralized with 2 M Tris–base.

Western blotting
Soluble extracts (S100) from HeLa cells were prepared according to our laboratory's standard protocol (Gannon and Lane, 1987). The samples for Western blotting from insect cells were prepared by adding SDS sample buffer directly to the cell pellet. The pellet was then sonicated and boiled for 5 min. SDS–polyacrylamide gel electrophoresis was performed according to Laemmli (1970). Electrophoretic transfer of proteins to nitrocellulose was performed according to Towbin et al. (1979). The nitrocellulose membrane was blocked with a 20% (w/v) solution of dried skimmed milk for 1 h at room temperature followed by incubation with the different monoclonal antibodies. After washing with 1% NP40 in PBS, the blot was incubated with a 1 h at room temperature with alkaline phosphatase-conjugated rabbit anti-mouse immunoglobulin (diluted 1:500 in PBS containing 10% FCS). The blots were washed again and incubated with bromochloroindoyl phosphosphate and nitroblue tetrazolium substrate (Harlow and Lane, 1988). The reaction was stopped with 1% acetic acid. For Western blotting of the cell extract of Saccharomyces cerevisiae pombe, a biotinylated sheep anti-mouse antibody at 1:500 dilution was used. After incubating with horseradish peroxidase-conjugated streptavidin the blot was developed with the DMB (diaminobenzidine)/H₂O₂ substrate (Harlow and Lane, 1988).

Purification of the monoclonal antibodies
The antibodies were purified on a protein A–Sepharose CL-4B column according to standard protocol (Harlow and Lane, 1988).

Biotinylation of antibodies
Purified antibodies were biotinylated using biotin N-hydroxy-
molar ratio of biotin TV-hydroxysuccinimide to protein was 20:1.

**Immunoprecipitation**

Four 15 cm plates of CV-1 cells at 70% confluency were each labelled with 250 μCi of [35S]methionine overnight. The cells were washed once with PBS and removed with 5 mM EDTA in PBS at 37°C. They were washed again with 150 mM NaCl, 50 mM Tris-HCl, 5 mM EDTA, pH 8.0 (NET), and lysed in 1% NP40 in NET containing 1 mM-phenylmethylsulphonyl fluoride and 1 mM dithiothreitol. After spinning at 100,000 revs min⁻¹ in a TL-100.2 rotor in Beckman TL-100 ultracentrifuge for 30 min, the supernatant was preabsorbed with protein G-Sepharose 4 Fast Flow (Pharmacia) for 30 min at 4°C. The suspension was spun at 9000 revs min⁻¹ in a Sorval RC-5B centrifuge for 5 min and the supernatant was divided into four aliquots per plate. An equal volume of hybridoma tissue culture supernatant containing each test antibody was added and incubated overnight at 4°C. Anti β-galactosidase monoclonal antibody BG2 was used as a control. A 50 μl sample of protein G-Sepharose 4 Fast Flow was added to each tube and the incubation continued for 30 min at 4°C. The protein G beads were washed with 250 mM NaCl, 1% NP40 in NET and boiled in 5 min sample buffer. Half of the sample was applied to a 12.5% SDS-polyacrylamide gel. After electrophoresis at 100 V the gel was fixed in 50% trichloroacetic acid (TCA) for 15 min and incubated in Enhance (Du Pont) for 15 min. Enhance was removed and the gel was washed with water and dried. The autoradiogram was developed overnight at room temperature.

**Enzyme-linked immunosorbent assay (ELISA)**

For the competition assay, protein at a concentration of 10 ng ml⁻¹ obtained from pC10 was coated onto a 96-well plastic microtitre plate by overnight incubation at 4°C. The plate was blocked with 10% bovine serum albumin (BSA) in PBS for 3 h at room temperature. Unlabelled monoclonal antibodies at different concentrations were added and incubated overnight at 4°C. The wells were washed with 0.1% NP40 in PBS and 100 μl of biotinylated anti-PCNA antibody (8 ng ml⁻¹) was added. After incubation for 3 h at 4°C, streptavidin-horseradish peroxidase at 1:1000 dilution in PBS containing 1% BSA, was added for 15 min. Colour was developed with 3',3',5',5'-tetramethylbenzidine (TMB) and H₂O₂, and read on an ELISA reader (Molecular Devices, V₅₀max at 450 nm after stopping the reaction with 50 μl of 1 N H₂SO₄).

For the sandwich ELISA an extract of CV-1 cells was prepared in 1% NP40 in NET. Different affinity-purified monoclonal antibodies at 5 μg ml⁻¹ were added to the wells of a 96-well microtitre plate and incubated overnight at 4°C. After incubating with a 10% BSA blocking solution for 4 h at room temperature different dilutions of CV-1 cell extracts were added and incubated overnight at 4°C. The wells were washed with 0.1% NP40 in PBS and biotinylated anti-PCNA antibody was added and incubated for 3 h at 4°C. After repeating the above washing the wells were incubated with horseradish peroxidase-labelled streptavidin for 15 min at room temperature. After washing away the unbound streptavidin the colour was developed with TMB and H₂O₂ as before.

**Cell staining**

CV-1 cells were grown on coverslips in DMEM containing 10% FCS. They were fixed in 1:1 (v/v) mixture of acetone–methanol for 5 min at room temperature and air dried. They were incubated with hybridoma tissue culture supernatant containing anti-PCNA antibodies for 1 h at room temperature. FITC-conjugated rabbit anti-mouse antibody was then added and incubated for 1 h at room temperature. After washing away the unbound antibody the coverslips were mounted in Gelvatol (Monsanto chemicals) on glass slides and dried.

**Tissue staining**

Formalin-fixed paraaffin-embedded tissue sections were deparaffi-

ized in xylene and passed through a graded ethanol series. They were fixed in acetone for 5 min and incubated with 1% NF40 for 5 min. After adding the monoclonal antibody the sections were incubated overnight at 4°C in humidified chamber. After washing, the slides were incubated with FITC-conjugated rabbit anti-mouse antibody for 1 h at room temperature. A third layer of FITC-conjugated swine anti-rabbit antibody was sometimes used to enhance the staining. The slides were mounted in Gelvatol and dried.

**Results**

**Preparation and purification of fusion protein**

The aim of this study was to produce a library of monoclonal antibodies against native PCNA that would be useful immunological reagents for the further analysis of PCNA activity in vivo and in vitro. An obvious source of the antigen would be PCNA purified from eukaryotic cells, but the complexity of the purification procedure and poor yield of the protein encouraged us to explore alternative ways of obtaining the antigen without compromising its purity. We therefore investigated the possibility of using the rat PCNA cDNA in E. coli. The β-galactosidase–PCNA fusion protein produced by pC288 was found to be insoluble in PBS and Tris-HCl buffers. We therefore decided to subclone the cDNA of PCNA into pRI72T, since protein A fusion proteins obtained from this vector were reported to be soluble (Nilson et al. 1985).

The cDNA cloned in pRI72T lacked 33 nucleotides from the 3' end of the PCNA coding region and therefore the expected size of the protein A–PCNA fusion protein obtained from this construct would be approximately 54K (K=10⁻⁵ M). The actual fusion protein produced by pC2T was about 68K and was found, significantly, to be completely soluble. Since the fragment of protein A present in this vector was about 27K, the molecular weight of the fusion protein obtained in this study represents an overestimation of about 14K. This discrepancy in the molecular weight of the fusion protein could be due to the lack of a termination codon in the cDNA construct of PCNA resulting in the use of a translational termination codon present downstream from the multiple cloning site in pRI72T. The discrepancy may also be due, in part, to the previously reported anomalous behaviour of PCNA in SDS–polyacrylamide gels (Matsumoto et al. 1987). The recovery of the protein A–PCNA fusion protein was about 80%. The preparation was contaminated with a minor band of about 70K and by some lower molecular weight species. The small species reacted with alkaline phosphatase-conjugated rabbit IgG, suggesting that they were derived from proteolytic cleavage of the protein A-containing section of the fusion protein.

The PCNA fusion protein produced in pC10 had an additional 20 amino acids at its N terminus derived from the amino terminus of the bacteriophage T7 gene 10 protein. The protein produced by pC10 in p lys SBL21(DE3) cells was insoluble in PBS and Tris–HCl buffers. It was found, however, to be soluble in 8 M urea. To our surprise the fusion protein remained soluble at 1 mg ml⁻¹ when the urea concentration was gradually lowered by slow dialysis against PBS over a period of 16–24 h. The protein obtained by this simple inclusion-body solubilization procedure was at least 80% pure based on the Coomassie Blue staining of SDS–polyacrylamide gels (Fig. 1). This preparation was used without further purification.
Fig. 1. SDS–polyacrylamide gel electrophoresis of protein purified from *E. coli* transformed with the p ClO PCNA expression plasmid. Lane 1: total cell extract of p lys S BL21(DE3) cells containing the parent plasmid p T7.7. Lane 2: total cell extract of p lys S BL21(DE3) cells containing p ClO. Lane 3: purified PCNA fusion protein from p lys S BL21(DE3) cells containing p ClO.

Production of monoclonal antibodies against PCNA

The purified protein A–PCNA fusion protein was used to immunize six mice. Dilutions of sera were tested for their ability to detect PCNA on Western blots of HeLa S100 fraction. All the sera reacted strongly with a single 36K band. One mouse had a titre of 1:10000 in this assay and was used for the fusion. Of the 36 wells that initially contained anti-nuclear antibody in the cell-staining assay, 11 clones were established as stable anti-PCNA antibody-producing clones. All antibodies were positive on Western blots of HeLa S100 fraction where they reacted with a single band of 36K. Some antibodies reacted more strongly than others; in particular, PC3 and PC6 gave very strong reactions while PC7 reacted very weakly. Two antibodies, PC2 and PC10, also reacted with a faint band of about 120K besides reacting with 36K protein band (Fig. 2).

Characterization of monoclonal antibodies

A summary of the characteristics of the anti-PCNA monoclonal antibodies are listed in Table 1.

**Immunoprecipitation.** The antibodies were tested for their capacity to immunoprecipitate PCNA from a [35S]-methionine-labelled cell extract of CV-1 cells. Ten of the 11 antibodies were capable of immunoprecipitating PCNA from the cell extract (Fig. 3). The antibodies PC1 (lane 2), PC2 (lane 3), PC3 (lane 4), PC7 (lane 5), PC8 (lane 6), PC9 (lane 7) and PC10 (lane 11) immunoprecipitated substantial amounts of PCNA. The remaining antibodies reacted more weakly and PC11 (lane 12) appeared completely negative. The identity of PCNA in these immunoprecipitation experiments was confirmed by Western blotting the [35S]-methionine-labelled immunoprecipitated bands (data not shown). Equal loading of the gel can be observed from the intensity of the 68K band, present in all samples. No other obvious bands co-precipitated with PCNA in all lanes.

**Competition assays.** To determine whether the 11 different antibodies bind to sterically discrete epitopes they were examined in competition assays and sandwich ELISA. For the competition assays four of the antibodies were biotinylated (PC3, PC8, PC9 and PC10). The ability of all 11 unlabelled antibodies to block the binding of these four labelled antibodies to solid-phase PCNA was quantified. The results of this analysis using antibody PC3 as label are illustrated in Fig. 4 and the complete results are summarized in Table 2. Although this analysis is not complete a minimum of three and a maximum of four

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**Table 1. Summary of the characteristics of the new anti-PCNA monoclonal antibodies**

<table>
<thead>
<tr>
<th>Monoclonal antibodies</th>
<th>Isotype</th>
<th>HeLa</th>
<th><em>S. pombe</em> (yeast)</th>
<th><em>S. frugiperda</em> (insect)</th>
<th>Immunoprecipitation</th>
<th>Cell staining</th>
<th>Tissue staining</th>
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<tr>
<td>PC1</td>
<td>IgG1</td>
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<td>+</td>
<td>+</td>
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<tr>
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* Spodoptera frugiperda.
Fig. 3. Immunoprecipitation of [35S]methionine-labelled CV-1 cells with anti-PCNA antibodies. The antibodies were: lanes 1, BG2; 2, PC1; 3, PC2; 4, PC3; 5, PC4; 6, PC5; 7, PC6; 8, PC7; 9, PC8; 10, PC9; 11, PC10; 12, PC11.

Table 2. Competitive inhibition between all 11 anti-PCNA antibodies and biotinylated antibodies PC3, PC8, PC9 and PC10

<table>
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<th>Competing antibody</th>
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<tr>
<td></td>
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</tr>
<tr>
<td>PC1</td>
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<td>PC2</td>
<td>0.3</td>
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<td>PC3</td>
<td>0.8</td>
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<tr>
<td>PC4</td>
<td>–</td>
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<tr>
<td>PC5</td>
<td>4.0</td>
</tr>
<tr>
<td>PC6</td>
<td>0.8</td>
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<tr>
<td>PC7</td>
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<tr>
<td>PC8</td>
<td>0.3</td>
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<tr>
<td>PC9</td>
<td>–</td>
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<tr>
<td>PC10</td>
<td>0.1</td>
</tr>
<tr>
<td>PC11</td>
<td>0.4</td>
</tr>
<tr>
<td>BG 2</td>
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</table>

* Micrograms of competing antibody to give 50% inhibition of labelled antibody.
† Too high to be significant.

sterically separate epitopes on PCNA can be defined. PC1, PC2, PC3, PC5, PC8, PC10 and PC11 fall into one group in that they all block the binding of the PC3, PC8 and PC10 to PCNA, albeit with widely differing efficiencies. In particular, PC10 is a very good inhibitor of the binding of all three of these antibodies and indeed is more efficient at blocking PC3 and PC8 binding than the appropriate homologous antibody. This suggests that PC10 has a particularly high affinity for PCNA. PC9 clearly recognizes a second discrete epitope, since, apart from itself, none of the antibodies can inhibit its binding to PCNA. At least one and possibly two further epitopes are defined by PC4 and PC7, since these antibodies do not block the binding of any of the labelled antibodies to PCNA. The PC6 antibody further refines the epitope map, since it is able to block partially the binding of PC3 but not PC8 or PC10, implying that even in the first large group of competing antibodies some differences in the binding site on PCNA exist. A diagrammatic representation of these results is shown in Fig. 6 (below).

Sandwich ELISA. The biotinylated antibody PC9 was used to try and detect PCNA captured by other anti-PCNA antibodies in a sandwich ELISA. The antibodies that were able to form a sandwich were PC2, PC3, PC5, PC6, PC8, PC10 and PC11 (Fig. 5). The best pair of antibodies for the quantitative assay of PCNA by sandwich ELISA is probably PC8 (capture) and PC9 (label). PC9 could not detect PCNA captured by antibodies PC4, PC7 and PC1.

Immunofluorescence: nucleolar and nucleoplasmic PCNA staining. In the earlier studies of Celis (Celis and Celis, 1985) immunofluorescence of methanol-fixed human amnion cells was used to define a series of distinct staining patterns for PCNA that changed through the cell cycle. This complex set of staining patterns seen with polyclonal anti-PCNA antibodies represents the location of the insoluble fraction of PCNA and is localized at sites of DNA synthesis. The monoclonal antibodies 19A2 and 19F4 have been reported to give similar results (Ogata et al. 1987a; Madsen et al. 1987). We compared the staining patterns obtained with these two commercially available antibodies with the 11 new anti-PCNA antibodies on cultures of CV-1 monkey epithelial cells fixed in acetone–methanol or methanol alone. The PC7 antibody failed to stain these cells at all. The majority of the new antibodies showed the same patterns as seen with 19A2 and 19F4 (data not shown). Specifically, the antibodies PC1, PC2, PC3, PC5,
Fig. 5. Sandwich ELISA of PCNA with biotinylated PC9 as probe. Fixed concentration of different 'capture' antibodies were added to coat the plate. Different dilutions of CV-1 extract were then added to the plate and probed with labelled PC9.

Fig. 6. Diagrammatic representation of the epitope mapping results. Epitopes are represented by circles and overlaps imply stearic interference.

Fig. 7. Immunofluorescence staining of CV-1 cells with anti-PCNA antibodies: A, PC9; B, PC9; C, PC4. Bars: 10 μm (A and C); 30 μm (B).

PC6, PC8, PC10 and PC11, like 19A2 and 19F4, showed granular staining throughout the nucleus. In some cells the Celis pattern Sb Sc was evident in that the nucleoplasm was stained but the nucleoli were not. Other cells showed staining of both the nucleoplasm and nucleoli (pattern Sd Sc) and, finally, a fraction of the cells showed very weak staining of the nucleoplasm but strong staining of the nucleoli (pattern Sf). PC4 showed a subtle difference in that cells with the Sb Sc and Sd Sc patterns were clearly visible (Fig. 7C); however, no cells with the Sf pattern of predominantly nucleolar staining were detected. The most surprising reaction, however, was with the antibody PC9. This antibody stained only the nucleoli (Fig. 7A and B) of the CV-1 cells. Most cells were strongly positive for this nucleolar staining. There was no evidence
for any nucleoplasmic staining with PC9 although in a small fraction of the cells the nucleolar staining was weak. Tissue staining. These antibodies were also checked for their reactivity on formalin-fixed paraffin-embedded tonsil sections, as PCNA is potentially a useful marker for the histological detection of cells with proliferative potential. PC2, PC5, PC6, PC8, PC10 and PC11 were all able to react with PCNA in sections of human tonsil where they preferentially stained the nuclei of cell within the germinal centres. None of the other antibodies reacted with PCNA in these sections (Table 1). Some of the positive antibodies were used to stain sections of rectal carcinoma tissue. The antibodies PC2, PC10 and PC11 stained the proliferating tumour tissue in these sections (data not shown).

PCNA in insect cells as well as in S. pombe
Since we had used CV-1 cells and HeLa cells for screening the hybridoma fusion, we wanted to know whether these antibodies would also react with PCNA from insect cells and S. pombe cell extract on Western blots. Of the 11 antibodies tested five of them, namely, PC2, PC3, PC5, PC8 and PC10, gave a positive signal. They reacted with a 34K band on a Western blot in insect cells (Fig. 8A) and with a 36K band in S. pombe extract (Fig. 8B). The antibody PC10 reacted particularly strongly with both the insect and S. pombe extracts.

Discussion
Antibodies against proteins required for SV40 DNA replication, like T antigen and DNA polymerase α, are very useful in understanding the various steps involved in DNA replication (Smale and Tjian, 1986; Murakami et al. 1986). To understand the role of PCNA in DNA replication it is necessary to have monospecific antibodies against PCNA that will recognize different epitopes on native PCNA. The monoclonal antibodies 19A2 and 19F4 were raised against SDS-denatured rabbit PCNA (Ogata et al. 1987a; Tan et al. 1987). We therefore decided to express PCNA in bacteria, purify it under non-denaturing conditions and use it to raise antibodies. Consistent with the native state of our immunogen, 10 of the 11 antibodies produced were able to immunoprecipitate PCNA from cell extracts. Little is known about the role of PCNA in DNA replication. Although it increases the processivity of polymerase α it has not been shown to associate with the enzyme directly. It has been speculated that PCNA inhibits an inhibitor of DNA replication (Lee et al. 1989). Since these new antibodies are able to recognize all forms of native PCNA, they are powerful tools with which to study various proteins associated with PCNA and their effect on the activity of PCNA.

These antibodies recognized different epitopes on PCNA as can be seen from the competition assay and sandwich assay data. The antibody PC9 seems to recognize a completely discrete epitope. It did not compete with any other antibody and binds to PCNA captured by seven other antibodies (PC2, PC3, PC5, PC6, PC8, PC10 and PC11). When CV-1 cells were stained with PC9 only the nucleoli were positive. This distribution was seen in all the cells examined though in a fraction of the cells the staining was relatively weak. The PC4 antibody failed to stain selectively the nucleoli of any cells. The remaining antibodies showed a more conventional series of staining reactions, in that cells were identified in each of the categories designated by Celis and were similar to the patterns seen with the existing anti-PCNA reagents. Despite these very different staining patterns seen with PC9, and to a lesser extent PC4, the immunochemical evidence strongly suggests that all of these antibodies are highly specific for PCNA. PC9 reacts strongly and specifically with PCNA in immunoblotting, immunoprecipitation and ELISA assays. The sandwich immunoassays also show that the PC9 epitope is present on the same molecules as the epitopes recognized by PC2, PC3, PC5, PC6, PC8, PC10, and PC11. If all of the staining reactions are due to PCNA, then the results suggest that while PCNA is present in both the nucleolus and the nucleoplasm throughout the cell cycle, the epitopes available for antibody binding are different in the two compartments. The PC9 epitope is only present on the nucleolar form and is absent from or masked on the nucleoplasmic form. These surprising results suggest that earlier interpretations of the nuclear location of PCNA are...
flawed because the serological reagents used were only able to detect a fraction of the total PCNA.

Why should PCNA be present in the nucleoli throughout the cell cycle? Autoradiography using [3H]thymidine suggests that replication of the nucleolar DNA occurs at a defined stage late in S phase. This is consistent with the appearance of the Sf pattern seen with most anti-PCNA antibodies and of 'movement' of PCNA into the nucleolus at this stage in S phase. The nucleolus may represent a special site for DNA replication. Minute virus of mice (MVM) replicates in the nucleolus, and antibodies and the earlier reports of 'movement' of PCNA appearance of the Sf pattern seen with most anti-PCNA antibodies and the earlier reports of 'movement' of PCNA


RAPID COMMUNICATION

PROLIFERATING CELL NUCLEAR ANTIGEN (PCNA) IMMUNOLOCALIZATION IN PARAFFIN SECTIONS: AN INDEX OF CELL PROLIFERATION WITH EVIDENCE OF DEREGLATED EXPRESSION IN SOME NEOPLASMS

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SUMMARY

Proliferating cell nuclear antigen (PCNA) is a 36 kD nuclear protein associated with the cell cycle. A monoclonal antibody, PC10, that recognizes a fixation and processing resistant epitope has been used to investigate its tissue distribution. Nuclear PCNA immunoreactivity is found in the proliferative compartment of normal tissues. PCNA immunoreactivity is induced in lectin stimulated peripheral blood mononuclear cells in parallel with bromodeoxyuridine incorporation and the number of cells with PCNA immunoreactivity is reduced by induction of differentiation in HL60 cells. In non-Hodgkin’s lymphomas a linear relation between Ki67 and PCNA staining was demonstrated. These data suggest that in normal tissues and lymphoid neoplasms, PCNA immunolocalization can be used as an index of cell proliferation. However, in some forms of neoplasia including breast and gastric cancer and in vitro cell lines, the simple relation between PCNA expression and cell proliferation is lost. In some breast and pancreatic tumours there is apparent deregulation of PCNA with increased expression in tissues adjacent to the tumours. The over-expression in some tumours and in adjacent morphologically normal tissue may represent autocrine or paracrine growth factor influence on PCNA gene expression.

KEY WORDS—PCNA, cell proliferation, immunohistochemistry, growth factors, autocrine.

INTRODUCTION

Cell kinetic information may be a useful adjunct to histologically based tumour classifications and contribute to the understanding of a range of non neoplastic conditions.¹ Immunohistological methods of assessing cell proliferation have particular advantages over other techniques because of the maintenance of cellular and tissue architecture, the relative simplicity of the methodology and the rapidity of results; neither in vivo nor in vitro labelling is required and the use of radioactivity is avoided.¹,² Immunohistology would be even more useful if available antibodies to cell cycle related antigens were applicable to conventionally fixed and processed tissue. The one widely used antibody that recognizes a cell cycle related antigen, Ki67,
only works on cryostat sections of snap frozen material.

Auto-antibodies from patients with SLE have been noted to identify a nuclear antigen present in proliferating cells\(^3\) and have been employed as operational markers of cell proliferation.\(^4\) Such auto-antibodies are now known to recognize \textit{proliferating cell nuclear antigen} (PCNA), an evolutionarily highly conserved 36 kD acidic nuclear protein which is directly involved in DNA synthesis.\(^8\) The gene for human PCNA has recently been cloned.\(^9\) PCNA is regulated in a complex manner with the gene being transcribed efficiently in both quiescent and proliferating cells, but PCNA mRNA normally only accumulates in proliferating cells.\(^10\)

The absence of stable PCNA mRNA in quiescent cells is associated with the presence of intron 4 in the gene: removal of this intron leads to high levels of accumulation of PCNA mRNA in such cells.\(^11\) Accumulation of the PCNA mRNA and the synthesis of high levels of the protein is stimulated by growth factors, notably PDGF, but is not necessarily associated with DNA synthesis: PCNA will accumulate in the presence of hydroxyurea, which inhibits DNA synthesis.\(^12\)\(^-\)\(^14\)

Monoclonal antibodies that recognize PCNA have been reported to be of value in assessing cell proliferation using immunohistological methods, although these have usually required cryostat sections or specially prepared histological material.\(^5\)\(^,\)\(^6\)\(^,\)\(^15\)\(^,\)\(^16\) In this report we describe the application of a new antibody\(^17\) that recognizes PCNA in conventionally fixed and processed histological material and we assess the possible usefulness and limitations of this reagent in histopathology.

\textbf{MATERIALS AND METHODS}

\textit{Antibodies}

Monoclonal antibodies were generated to genetically engineered rat PCNA using conventional methods.\(^17\) Of the eleven clones generated with anti PCNA specificity, six were found to react with formalin fixed histological material, and one clone designated PC10 was chosen for further detailed study because of its highest avidity in an ELISA assay. Supernatant from clone PC10 was used at a range of dilutions.

\textit{Immunohistochemistry}

Conventional fixed and processed normal human tissue (listed in Table I), breast cancers, pancreatic tumours and non-Hodgkin’s lymphomas were studied. Sections (4 \(\mu m\)) were cut, mounted on poly L-lysine coated glass slides and air-dried overnight at room temperature. Sections were de-waxed, taken through alcohol and then immersed for 10 min in 25 per cent phosphate-buffered saline in methanol with 0-5 per cent hydrogen peroxide to block endogenous peroxidase activity. Sections were subsequently taken to water and immunostaining was performed using the ABC method (Dakopatts UK Ltd) with primary incubations for 1 h or 18 h (i.e. overnight) at a range of dilutions. A dilution of 1:200 with overnight incubation was found to be optimal. Diaminobenzidine–hydrogen peroxide was employed as a chromogen and a light haematoxylin counterstain was used. The nature of staining including its spatial distribution within cells was assessed, and in some cases absolute counts of PCNA immunoreactivity were made by scoring a minimum of 1000 cells. The distribution of PCNA immunoreactivity within tissues was also recorded.

\begin{table}[h]
\centering
\begin{tabular}{|l|}
\hline
\textbf{Normal tissues examined for PCNA immunoreactivity} \\
\hline
Skin \\
Hair bulb \\
Sweat gland \\
Oesophagus \\
Stomach \\
Small intestine, including Brunner's glands \\
Large intestine \\
Pancreas \\
Gall bladder \\
Liver \\
Thymus \\
Lung \\
Adrenal \\
Thyroid \\
Cerebrum \\
Cerebellum \\
Peripheral nerve \\
Cardiac muscle \\
Smooth muscle \\
Skeletal muscle \\
Kidney \\
Bladder \\
Prostate \\
Bone marrow \\
Endometrium, proliferative and secretory phase \\
Ovary \\
Testis \\
Tonsil \\
Lymph node \\
\hline
\end{tabular}
\end{table}
To investigate the effects of fixation, clinical material that had been fixed in a range of reagents (buffered and unbuffered formalin, mercury based fixatives, methacarn and Bouin's) was examined. In addition small intestine from adult Wistar rats was fixed in neutral buffered formal saline for 6, 24, 48, 72, and 96 h and then processed to paraffin. Material from a clinical colectomy specimen was fixed in 10 per cent formalin for 12, 24, 48, 72 and 96 h and then similarly processed. The effect of decalcification by EDTA or weak acid was assessed on clinical material.

Cryostat sections from cases of reactive lymphoid tissue or lymphoma were stained for the proliferation marker Ki67 in parallel with PC10 staining of fixed and wax embedded material from the same cases. Cryostat sections of tonsil, breast tumour and xenografts of pancreatic carcinoma cell lines were also stained with PC10 after fixation in either acetone, acetone/methanol or formaldehyde.

**Cell culture**

Human epidermal keratinocytes were grown on glass coverslips as previously described. When small colonies had formed after 4 to 6 days, cultures were pulse labelled for 1 h with tritiated thymidine, fixed in 1:1 acetone/methanol for 5 min and stained for PCNA by the indirect immunoperoxidase method with a peroxidase labelled anti-mouse antibody (Dakopatts UK Ltd). Slides were then dipped in photographic emulsion and autoradiograms produced. Several human pancreatic carcinoma cell lines were grown on multiwell glass slides in RPMI 1640 with 10 per cent fetal calf serum as described elsewhere (Hall et al. in preparation). After fixation in 1:1 acetone/methanol and air drying, cells were stained by the indirect immunoperoxidase method for Ki67 (Dakopatts UK Ltd) and for PCNA.

Human peripheral blood mononuclear cells were prepared by differential cytocentrifugation on Ficoll–Hypaque and aliquots were treated with phytohaemagglutinin (Sigma) at 0.5 μg/ml or left untreated as controls. Cells were incubated in 96-well micro-titre plates at 37°C in a humidified CO2 incubator. At various time points cells were labelled with bromodeoxyuridine for 1 h. Cytocentrifuge slides were prepared and stained for bromodeoxyuridine using the Amersham Cell proliferation kit (Amersham UK Ltd) and PCNA by the indirect immunoperoxidase method.

The myelomonocytic cell line HL60 (obtained from ICRF) was treated with phorbol esters (10 nM 12-O-tetradecanoyl phorbol 13-acetate [TPA], Sigma) to induce macrophage differentiation. Cytocentrifuge preparations were prepared from control untreated cultures or from cultures treated with TPA for 72 h, fixed in 1:1 acetone/methanol for 5 min at room temperature and air dried. Cells were then stained for PCNA or Ki67 by the indirect immunoperoxidase method.

**Flow cytometry**

HeLa cells grown in suspension culture in RPMI 1640 containing 5 per cent fetal calf serum were fixed in alcohol acetone (1:1) for 5 min, spun down and washed in phosphate buffered saline (PBS). They were stained by the indirect immunofluorescence method with PC10 and a series of control antibodies. After incubation for 30 min on ice and two washes in PBS, the cells were incubated for a further 30 min in fluorescein linked rabbit anti-mouse F(ab)2 antibody (Dakopatts UK Ltd). After two further washes in PBS, cells were stained in a solution containing 50 μg/ml propidium iodide in the presence of 1 mg/ml RNAase (both from Sigma UK Ltd). Red fluorescence from the propidium iodide was then measured simultaneously with green fluorescence from the antibody staining using a FACScan flow cytometer (Becton Dickinson, California). Fluorescence in a minimum of 10,000 cells was measured and the results stored on a Consort 30 computer, allowing subsequent data analysis using the Lysis software.

**RESULTS**

**Distribution of PCNA immunoreactivity in histological sections of normal adult tissues**

PC10 staining is almost entirely confined to the nucleus and may show a diffuse or granular pattern or a mixture of both. Rarely cytoplasmic staining is observed: the nature of this is unclear but it may represent cytoplasmic synthesis or breakdown. Mitotic cells commonly show diffuse staining throughout the cell, but this is not surprising as the nuclear membrane is lost during mitosis. Some mitotic cells fail to show any PC10 staining. In this study all nuclear staining was considered as positive, regardless of its nature.

The distribution of nuclear PC10 staining in non-neoplastic histological material is entirely consistent with PCNA being associated with cell proliferation (see Fig. 1). Staining is seen in those tissues known from other studies to be actively proliferating, and
moreover the spatial distribution of staining in those tissues is as would be expected. For example, staining is seen in germinal centres and scattered cells in the paracortex of lymphoid tissue; it is present in the basal layer of stratified squamous epithelia and in the majority of cells in the hair bulb. Nuclear PCNA is present in the proliferative compartments of stomach, small intestine and colon. In the small intestine, weak diffuse PCNA staining is observed above the generally accepted zone of proliferation within the crypt. This staining diminishes as cells progress up to the villus and is then lost. PCNA immunoreactivity is present in epithelial and stromal cells of proliferative phase endometrium, but is not seen in late secretory phase endometrium. In the testis the majority of spermatogonia are stained but spermatids and sperm are unstained as are interstitial cells and Sertoli cells. In the ovary, ova arrested in meiosis show PCNA staining both in the nucleus and in the cytoplasm. This observation is consistent with the report of Zuber et al. that PCNA is stockpiled during oogenesis for use in early embryogenesis.

In those tissues known to be non-proliferative or to show only low turnover, PCNA immunoreactivity is minimal. For example staining is not seen in the adult central or peripheral nervous system, nor in skeletal, smooth nor cardiac muscle, nor is it present in normal hepatocytes, although rare Kupffer cells show nuclear immunoreactivity. Very little staining is seen in normal adult kidney. In the normal pancreas rare acinar and duct cells show PC10 staining consistent with observations made on cryostat sections of normal human pancreas stained with Ki67 (Hall & Lemoine unpublished). In endocrine tissues only rare PC10 staining cells are identified, but it should be noted that in the adrenal those cells that stain are in the zona glomerulosa as expected.

**Effects of section preparation and fixation**

PCNA immunoreactivity is greatly reduced or abolished if cut sections are heated to assist adherence to glass slides. The reason for this remains unclear. PCNA immunoreactivity can be seen after fixation in a wide range of solutions including formalin (buffered and unbuffered), methacarn and Bouin's reagent. In all cases, except methacarn, both diffuse and granular nuclear staining can be identified. With methacarn fixation only granular staining is seen. The time of fixation can greatly alter the ability to identify PCNA immunoreactivity. Staining in rat small intestine and human colon is greatly reduced after 48 h fixation and is virtually abolished after 72 h. Similar observations have been made in other clinical material (Foulis and Hall, unpublished observations). Protease digestion is not required to unmask the antigen; in fact, protease digestion of sections abolishes subsequent staining. PCNA immunoreactivity can be detected after decalcification by chelating agents or weak acids, but as with fixation, prolonged exposure greatly diminishes staining.

**Staining of cryostat sections**

Staining of cryostat sections with PC10 gives poor results irrespective of section fixation. Diffuse weak cytoplasmic staining may be identified but the clear nuclear localization observed after conventional fixation and processing to paraffin is not seen.

**Correlation with other parameters of cell proliferation**

**Staining of cultured cells**—PCNA immunoreactivity was generally confined to the nucleus although some cytoplasmic staining was also seen. Cultures of human epidermal keratinocytes are in a relatively hyperproliferative state with many (but not all) cells taking up thymidine during a 1 h pulse as shown by autoradiography. Nuclear PCNA immunoreactivity was observed in a similar proportion of keratinocytes and double labelling experiments showed a general concordance between PCNA immunoreactivity and autoradiographically demonstrated thymidine incorporation. While all thymidine incorporating keratinocytes appeared to...
show PCNA immunoreactivity, occasional cells showed PCNA immunoreactivity but no thymidine incorporation. This observation is consistent with PCNA being expressed in phases of the cell cycle other than S phase.

Human peripheral blood mononuclear cells showed only very rare (< 1 per cent) PCNA immunoreactive cells, consistent with observed low rates of bromodeoxyuridine incorporation. After phytohaemagglutinin stimulation, blast transformation occurs in the majority of cells by 48 h as judged by morphology and bromodeoxyuridine incorporation. There was a similar dramatic increase in the number of PCNA immunoreactive cells as shown in Fig. 2. The highly proliferative cell line HL60 can be induced to differentiate towards macrophages by treatment with phorbol esters, the differentiated cells exiting the cell cycle. A dramatic reduction of the number of HL60 cells staining with Ki67 after treatment with TPA was mirrored by a reduction in the number of PCNA immunoreactive cells (Table II).

In cultures of established tumorigenic and immortalized cells such as pancreatic carcinoma cell lines, a high proportion of cells, typically 70–90 per cent, show Ki67 immunoreactivity. In these cell lines PCNA staining was present in all cells observed both in culture and as tumour xenografts. This observation is consistent with the results of flow cytometric analysis of HeLa cells where all cells showed PCNA and there was no relationship with the cell cycle as judged by DNA content (Fig. 3). It should be noted that HeLa and the other tumour cell lines studied are very fast cycling cells and the half life of PCNA is long.21

Clinical material—The number of PCNA staining cells was compared with the number of Ki67 staining cells in 20 lymph nodes comprising four cases of reactive lymphoid hyperplasia and 16 cases
PROLIFERATING CELL NUCLEAR ANTIGEN

There is a linear relationship between the number of cells with PCNA expression and the number staining with Ki67 in non-Hodgkin’s lymphomas.

Fig. 3—There is a linear relationship between the number of cells with PCNA expression and the number staining with Ki67 in non-Hodgkin's lymphomas.

Fig. 4—Panel A shows the DNA histogram for HeLa cells, with a G1/G0 peak, S-phase region and G2 peak. Panel B shows DNA staining on the abscissa as in panel A on a linear scale against negative control antibody staining plotted on a log scale (four decades). In panel C, the result with PC10 is shown, in which 98 per cent of cells show strong labelling.

A linear relationship between Ki67 and PCNA staining was observed with a correlation coefficient of 0.91 (Fig. 4).

Other clinical material is the subject of separate studies but in gastric carcinoma and haemangio- pericytomas there is a lack of correlation between PCNA index and S phase fraction as assessed by flow cytometry, but a correlation of number of PCNA immunoreactive cells with prognosis: higher PCNA counts tend to be related to poorer prognosis. In contrast, preliminary data on breast cancer (Barnes et al. unpublished) indicates no correlation between PCNA index and prognosis or other indices of proliferation.

In several studies, the number of PCNA immunoreactive cells within tumours exceeded that expected. Furthermore, in 6 of 16 cases of pancreatic tumour examined, there was a dramatic increase in the number of PCNA immunoreactive cells in surrounding, histologically normal, parenchyma. Four of the tumours were ductal adenocarcinomas and two were islet cell tumours (insulinomas).
Similarly, examination of histologically normal breast tissue adjacent to carcinoma of the breast showed, in 17 of 23 cases, dramatically increased PCNA expression (Fig. 5).

**DISCUSSION**

Proliferating cell nuclear antigen (PCNA) has also been known as cyclin24,25 or as auxiliary protein for DNA polymerase δ.26-28 Microinjection of antibodies to PCNA29 and the use of anti-sense oligonucleotides30 suggest that PCNA is an essential requirement for DNA synthesis and has also been shown to be required for leading strand synthesis in SV40 virus replication.31 PCNA functions as a co-factor for DNA polymerase δ in DNA synthesis, but may also be involved in unscheduled DNA synthesis.32,33 PCNA is highly conserved throughout phylogeny, being present in plants, yeast and higher eukaryotes.33 PCNA has been reported to be a cell-cycle regulated protein when examined by immunofluorescence.7

Immunofluorescent studies of cultured cells have indicated that there are two populations of PCNA present during S phase:21 nucleoplasmic PCNA corresponds to the PCNA present at low levels in quiescent cells that are capable of cell division, and is not apparent in cells fixed in organic solvents such as methacarn. The second form of PCNA is associated with sites of DNA replication and cannot be extracted with organic solvents. This may explain our observation that only granular staining is seen in methacarn fixed tissues representing replication site associated PCNA. Differences between some reports on the use of autoantibodies and monoclonal antibodies may reflect differences in the epitopes recognized.4,5,6,16,36 Differences in section preparation and fixation may also be of importance.

Recent data suggest that there are only small changes in the total level of PCNA in the cell cycle (perhaps only two to three fold), but there is a dramatic alteration in the proportion associated with replication sites, particularly in S phase.37 The amount of PCNA present in the nucleus of cycling cells appears to be greater than that required for DNA synthesis.37 In long-term quiescent cells PCNA is undetectable by immunoblotting methods, but it is present at low levels (10 per cent that in cycling cells) in cells capable of division. A final point of note is that the half-life of PCNA protein is about 20 h21 and thus it may be immunologically detectable in cells that have recently left the cell cycle. The data presented in this current report are largely in accord with these biochemical and cell biological observations.

The spatial distribution of PCNA immunoreactive cells in normal proliferating tissues is exactly as one would expect with a marker of cell proliferation. That occasional mitoses do not stain cannot at present be explained. The possibility that these relate to artefacts of fixation or processing seems unlikely since stained and unstained mitoses can be seen within the same section, sometimes in close proximity. The presence of weak staining above the generally accepted highest point in gastrointestinal crypts for proliferating cells probably reflects the relatively long half-life of the PCNA protein and the rapid migration of gastrointestinal cells. These simple observations from histological material are supported by the cell biological experiments presented here. Of particular importance are the induction of PCNA immunoreactivity in phytohaemagglutinin stimulated human peripheral blood mononuclear cells in parallel with bromodeoxyuridine labelling, and the reduction of staining of HL60 cells with PC10 and Ki67 that occurs in parallel after induction of macrophage differentiation by phorbol esters. Similar results have been reported using auto-antibodies.3,7 Finally, the observation that there is a linear relationship between the number of cells showing PCNA and the number showing Ki67 immunoreactivity in nodal lymphoid neoplasms, or PCNA immunoreactivity and the S phase fraction as determined by flow cytometry in gastrointestinal lymphomas,38 also supports the notion that PCNA immunoreactivity is a marker of cell proliferation in fixed histological material. This is further supported by recent data from Alison et al. (in preparation) where there is a close spatial and numerical correlation between PCNA immunoreactivity and thymidine incorporation into normal rat tissues and those stimulated to proliferate by exogenous factors, such as isoproterenol treatment which induces proliferation in the salivary gland.

In contrast to these data supporting the notion that PCNA expression can be used as an operational marker of cell proliferation, a number of other results require further consideration. Firstly, there is an apparent difference between primary cells (e.g. keratinocytes and peripheral blood monocytes) and established cell lines. Peripheral blood monocytes and keratinocytes show a close relationship between PCNA immunoreactivity and other parameters of cell proliferation. In contrast this is not apparent
with established cell lines. This may simply reflect the relatively long half-life of the PCNA protein or may require a more complex explanation. Secondly, although there is a linear correlation between Ki67 and PCNA immunoreactivity in nodal lymphoma and between S phase fraction and PCNA staining in gastrointestinal lymphoma, recent studies of gastric cancer, breast cancer (Barnes et al. unpublished) and of haemangiopericytomas have shown that the correlation between S phase fraction and PCNA immunoreactivity is very poor. In all these situations there appear to be more PCNA immunoreactive cells than would be expected.

The final intriguing observation is that in histopathologically normal tissues adjacent to tumours there is in some, but not all cases, a dramatic increase in immunohistologically detectable PCNA-containing cells (Fig. 5). This has been observed in breast lobules adjacent to breast tumours, as well as in pancreatic exocrine parenchyma adjacent to exocrine and endocrine tumours of the pancreas. PCNA expression is regulated at both the transcriptional and post-transcriptional level, in particular by alterations in mRNA stability. Recent evidence suggests that growth factors can induce increased PCNA mRNA stability and consequently PCNA expression. Our observations might then be explained by postulating that some of the tumours are actively secreting PDGF, or similar growth factors, that are stabilizing the PCNA mRNA and thus inducing PCNA protein accumulation in the surrounding normal cells without necessarily inducing DNA synthesis. Such a hypothesis is consistent with observations made in uinaemic mice where recruitment of surrounding cells by factors elaborated by tumour cells is seen to occur. Autocrine and paracrine growth factor mediated regulation of PCNA expression may also explain the excess of PCNA immunoreactive cells seen in certain tumours.

In conclusion, we have demonstrated, firstly, that PC10 immunostaining can demonstrate the proliferative compartment of conventionally fixed and processed normal tissues. Secondly, the interpretation of PCNA immunoreactivity requires careful consideration of the length of fixation of the tissues and the way in which sections are prepared. Finally, there remain some doubts as to the relationship between PCNA expression and cell proliferation in the context of, at least some forms of, neoplasia. It may be that this can be explained, in part, by the long half-life of PCNA. In addition it is possible that the control of PCNA expression may be deregulated, within and adjacent to some types of tumour, perhaps by the autocrine secretion of growth factors.

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