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 fulfill 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 (Jaakulski 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 (Prelich 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; Matsumoto et al. 1987; Almendral et al. 1987; Ogata et al. 1985). The gene for PCNA is present not only in mammals (Matsumoto 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. 1987a).
1987b). 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 at least three distinct epitopes on primate PCNA. Several of these antibodies were able to immunoprecipitate the native protein and recognize it in different species including Schizosaccharomyces pombe and Spodoptera frugiperra (fall armyworm) IFLB-SF21 cells (insect cells). Immunocytochemical studies of PCNA using these new antibodies suggest that the protein exists in multiple states in the cell nucleus. The results help to resolve some of the complexities of the cell staining pattern observed with polyclonal anti-PCNA sera. Several of the antibodies were able to react with PCNA in routine formalin-fixed histological sections, implying that they may be useful reagents for the analysis of the proliferative status of tumour tissues.

Materials and methods

Preparation of cDNA constructs

The cDNA of rat PCNA, PCR-1 in pBR322 was obtained from Morisuki (Matsumoto 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 Xbal. An Xbal and HinII digestion of PCR-1 gave two fragments of 1.0 and 1.1 kb (1 kb = 10^5 base-pairs). The 1.0 kb fragment containing the PCNA cDNA was purified and ligated into Xbal and the blunt-ended pUR288 vector to give pC288. A second construct was made in the protein A expression vector pHITT2T obtained from Pharmacia (Nilson et al. 1985). A 728 bp (base-pair) fragment containing part of the PCNA cDNA in pC288, obtained by BamHI and PstI digestion, was subcloned into pHITT2T to give pC288T. This construct lacked the 3'-end 93 nucleotides of PCNA.

Preparation of cDNA constructs

The samples for Western blotting from insect cells were prepared according to our laboratory’s standard protocol (Gannon and Lane, 1987). The samples for Western blotting from insect cells were prepared according to our laboratory’s standard protocol (Gannon and Lane, 1987).

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 according to our laboratory’s standard protocol (Gannon and Lane, 1987). The samples for Western blotting from insect cells were prepared according to our laboratory’s standard protocol (Gannon and Lane, 1987).

Purification of the fusion proteins

Protein A fusion protein. A bacterial culture of E. coli N4830-1 cells containing pC2T was grown and induced according to the manufacturer’s instructions. The cells were pelleted at 7000 revs min^-1 in a GSA 600 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 30 000 revs min^-1 in a 50Ti rotor in a Beckman L6-S5M centrifuge for 20 min. The supernatant was applied to a column of human IgG-Sepharose 4 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.

77 fusion protein. A culture of pC10 in E. coli p lys S (BL21DE3) cells was grown and induced as previously described (Studier and Moffatt, 1986). The cells were pelleted at 7000 revs min^-1 and frozen rapidly. Thawing the pellet in 50 mM Tris–HCl, 1 mM EDTA, pH 8.0, released intracellular lysosome resulting in the lysis of the cells. The lysed cells were sonicated for 20 s and spun at 30 000 revs min^-1 for 30 min (as above). The pellet was washed twice with 1% NP40 and stirred in 1 M-urea for 1 h at 4°C and then centrifuged at 30 000 revs min^-1 for 30 min. The pellet was resuspended in 8 M urea, spun at 30 000 revs min^-1 for 30 min and the supernatant dialysed gradually against PBS to yield a soluble PCNA fusion protein.

Monoclonal antibody production

At intervals of 21 days six mice were injected intraperitoneally with 50 μg of protein A–PCNA fusion protein (obtained from pC2T) in PBS emulsified with an equal volume of Freund’s complete adjuvant. After three injections the titres of the sera were monitored by Western blotting and the mouse with the highest titre was boosted with two injections of 10 μg of PCNA fusion protein obtained from pC10. These final boosts were carried out on two successive days: one intraperitoneally and the other intravenously. Three days after the final intravenous boost the mouse was killed and the spleen removed. The spleen cells were fused with mouse myeloma cells Sp2/0-Ag14 using 50% polyethylene glycol 1500 (Harlow and Lane, 1988). The cells were grown in Dulbecco’s modified Eagle’s medium (DMEM) containing 20% foetal calf serum (FCS) and supplemented with interleukin 6 at 4 ng ml^-1 as growth factor (E. Weiss, personal communication). Hypoxanthine and azaarase were used to select specifically the hybridoma cells. The fusion was screened by cell staining of CV-1 cells (an immortalized line of monkey kidney epithelial cells) and Western blotting of HeLa S-100 fraction (see below).

Isotyping

The antibodies were isotyped using the Serotec Mouse Isotyping Kit (Serotec, Oxford, UK).

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 according to our laboratory’s standard protocol (Gannon and Lane, 1987). The samples for Western blotting from insect cells were prepared according to our laboratory’s standard protocol (Gannon and Lane, 1987).
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 7000 revs min\(^{-1}\) in a Sorvall 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 for 5 min in 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 μg/ml was boiled in SDS—polyacrylamide gel 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.

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 expressing 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 prIT2T, since protein A fusion proteins obtained from this vector were reported to be soluble (Nelson et al. 1985).

The cDNA cloned in prIT2T lacked 93 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=10^3 M\(_{r}\)). 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 over-estimation 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 prIT2T. 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 S BL21(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 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.

Proliferating cell nuclear antigen
Fig. 1. SDS–polyacrylamide gel electrophoresis of protein purified from E. coli transformed with the pC10 PCNA expression plasmid. Lane 1: total cell extract of p lys S BL21(DE3) cells containing the parent plasmid pT7.7. Lane 2: total cell extract of p lys S BL21(DE3) cells containing pC10. Lane 3: purified PCNA fusion protein from p lys S BL21(DE3) cells containing pC10.

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 1:10 000 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 8), PC8 (lane 9) 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

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>S. pombe (yeast)</th>
<th>S. frugiperda* (insect)</th>
<th>Immunoprecipitation</th>
<th>Cell staining</th>
<th>Tissue staining</th>
</tr>
</thead>
<tbody>
<tr>
<td>PC1</td>
<td>IgG1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>–</td>
</tr>
<tr>
<td>PC2</td>
<td>IgG1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC3</td>
<td>IgG2b</td>
<td>+</td>
<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>PC4</td>
<td>IgG2b</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
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<td>IgG1</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>+</td>
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<td>+</td>
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<tr>
<td>PC7</td>
<td>IgG1</td>
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<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PC8</td>
<td>IgG3</td>
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<td>–</td>
<td>–</td>
<td>+</td>
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<td>IgG1</td>
<td>+</td>
<td>–</td>
<td>–</td>
<td>+</td>
<td>–</td>
<td>–</td>
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<tr>
<td>PC10</td>
<td>IgG2a</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
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<td>PC11</td>
<td>IgG1</td>
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<td>–</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
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</table>

* 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>
<thead>
<tr>
<th>Competing antibody</th>
<th>PC3</th>
<th>PC8</th>
<th>PC9</th>
<th>PC10</th>
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<tbody>
<tr>
<td>PC1</td>
<td>0.4</td>
<td>1.9</td>
<td>–</td>
<td>2.1</td>
</tr>
<tr>
<td>PC2</td>
<td>0.3</td>
<td>1.0</td>
<td>–</td>
<td>0.2</td>
</tr>
<tr>
<td>PC3</td>
<td>0.8</td>
<td>1.2</td>
<td>–</td>
<td>1.6</td>
</tr>
<tr>
<td>PC4</td>
<td>4.0</td>
<td>30.0</td>
<td>–</td>
<td>25</td>
</tr>
<tr>
<td>PC5</td>
<td>0.8</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PC6</td>
<td>–</td>
<td>–</td>
<td>–</td>
<td>–</td>
</tr>
<tr>
<td>PC7</td>
<td>0.3</td>
<td>2.0</td>
<td>–</td>
<td>0.9</td>
</tr>
<tr>
<td>PC8</td>
<td>–</td>
<td>–</td>
<td>0.4</td>
<td>–</td>
</tr>
<tr>
<td>PC9</td>
<td>0.1</td>
<td>0.3</td>
<td>0.1</td>
<td>–</td>
</tr>
<tr>
<td>PC10</td>
<td>0.4</td>
<td>1.2</td>
<td>–</td>
<td>0.4</td>
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<tr>
<td>BG2</td>
<td>–</td>
<td>–</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, PC6, PC9 and PC10 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 PC11.

Immunofluorescence: nucleolar and nucleoplasmic PCNA staining. In the earlier studies of Celis (Celis and Celis, 1986) 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,
Antigen dilution

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.

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/Se) and, finally, a fraction of the cells showed very weak staining of the nucleoplasm but strong staining of the nucleoli (pattern Sc). PC4 showed a subtle difference in that cells with the Sb/Sc and Sd/Se patterns were clearly visible (Fig. 7C); however, no cells with the Sc 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

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).
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 a potentially 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. 1989). 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) and are unable to immunoprecipitate native PCNA or inhibit PCNA-dependant DNA replication (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 more 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 δ 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 immunochemo 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 the earlier reports 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 nucleolar components are redistributed during adenovirus infection (Walton et al. 1989). SV40 DNA is associated with the nucleoli during SV40 infections (Geukenks and May, 1974). Alternatively, it is possible that PCNA may play another discrete, non-replicative role in the nucleolus. In this context it is interesting to note that two other proliferative markers, the Ki-67 antigen (Verheijen et al. 1989) and numatrin (Feuerstein et al. 1988), are also nucleolar and that the rapid onset of ribosomal RNA transcription is a characteristic early feature of the mitotic response following recovery from amino acid starvation (Grummt et al. 1976). Perhaps PCNA, like several other replication factors (DePamphilis, 1988), may also be active in transcription.

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