

Drosophila Proliferating Cell Nuclear Antigen

STRUCTURAL AND FUNCTIONAL HOMOLOGY WITH ITS MAMMALIAN COUNTERPART*

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A protein with an apparent mass of 36 kDa was purified from *Drosophila melanogaster* embryos using a protocol developed for the purification of proliferating cell nuclear antigen (PCNA) from human 293 cells. The *Drosophila* protein comigrated with human PCNA on one-dimensional sodium dodecyl sulfate-polyacrylamide gels and cross-reacted with monoclonal anti-rabbit PCNA antibodies. NH₂-terminal amino acid sequence analysis revealed that the putative *Drosophila* PCNA was highly homologous to human PCNA. Of the first 22 amino acids, 16 were identical, and 4 of the remaining 6 were changed conservatively. Results of total amino acid analysis were also consistent with a high degree of similarity between *Drosophila* PCNA and human PCNA. Functional analysis using the reconstituted simian virus 40 *in vitro* DNA replication system demonstrated that *Drosophila* PCNA could substitute, albeit with reduced efficiency, for human PCNA in stimulating simian virus 40 DNA synthesis. Affinity-purified anti-*Drosophila* PCNA antibodies cross-reacted with human PCNA and were able to recognize specifically *Drosophila* PCNA both on crude homogenate immunoblots and by indirect immunofluorescence analysis of proliferating cells in larval tissues *in situ*. These antibodies thus promise to be useful probes for the study of cell proliferation in this rapidly developing organism.

Proliferating cell nuclear antigen (PCNA),¹ as its name implies, is found in high concentrations only in actively dividing cells (for recent reviews, see Celis *et al.*, 1987; Tan, 1989). Originally recognized as an autoimmune antigen in a subset of human patients with systemic lupus erythematosus (Miyachi *et al.*, 1978), it has now been identified as a highly conserved protein in a number of widely divergent species (see *e.g.* Bravo and Celis, 1980; Bravo *et al.*, 1981; Mathews *et al.*, 1984; Bauer and Burgers, 1988; Olins *et al.*, 1989).

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¹ The abbreviations used are: PCNA, proliferating cell nuclear antigen; SV40, simian virus 40; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; RF, replication factor.

Interest in PCNA was sparked recently by recognition of its essential role in SV40 DNA replication *in vitro* (Prelich *et al.*, 1987a; Prelich and Stillman, 1988). It was proposed that PCNA acts as an auxiliary factor for DNA polymerase δ , facilitating processive leading strand DNA synthesis by this enzyme (Tan *et al.*, 1986; Bravo *et al.*, 1987; Prelich *et al.*, 1987b). A role for PCNA in host chromosomal replication has also been suggested based both on results of *in vivo* antibody microinjection studies (Zuber *et al.*, 1989) and *in situ* immunocytochemistry (see *e.g.* Celis and Celis, 1985; Bravo and Macdonald-Bravo, 1985, 1987; Olins *et al.*, 1989). However, this role is not yet proven.

We expected that *Drosophila melanogaster* would possess a protein homologous to mammalian PCNA. Unlike any vertebrate, *Drosophila* is a higher eukaryote suited to systematic genetic manipulation. If *Drosophila* PCNA could be identified, there would be the potential to demonstrate directly by genetic means the postulated role of PCNA in eukaryotic chromosomal replication. In addition, PCNA might represent an ideal marker to correlate cell proliferation with differentiation in this rapidly developing organism.

In this paper, we report the identification and purification of *Drosophila* PCNA. Analyses of NH₂-terminal protein sequence and total amino acid composition suggest that it is structurally homologous to mammalian PCNA. Functional studies indicate that *Drosophila* PCNA can substitute, albeit with reduced efficiency, for human PCNA in a reconstituted SV40 DNA replication system *in vitro*. Polyclonal antibodies raised against *Drosophila* PCNA promise to be useful both in developmental studies and for immunocytochemistry.

EXPERIMENTAL PROCEDURES

Materials—The sources of most of the materials were as described previously (Smith *et al.*, 1987). [α -³²P]dATP was from Du Pont-New England Nuclear.

Antibodies—Affinity-purified goat anti-rabbit IgG and goat anti-mouse IgG were from Cappel Laboratories (Cochranville, PA). Rhodamine-conjugated affinity-purified donkey anti-rabbit IgG was from Jackson ImmunoResearch (West Grove, PA). Rabbit anti-*Drosophila* PCNA antibodies and anti-*Drosophila* lamin antibodies were affinity purified according to Fisher and Smith (1988). Monoclonal anti-rabbit PCNA antibodies 19A2 and 19F4 were as described (Ogata *et al.*, 1987). Rabbit anti-DNA topoisomerase II antiserum was as specified previously (Berrios *et al.*, 1985) and was the generous gift of Dr. Neil Osheroff (Vanderbilt University, Nashville, TN).

Purification of *Drosophila* PCNA—*D. melanogaster* (Oregon R, P2 strain) were grown in mass culture according to Allis *et al.* (1977). PCNA was purified from 30–50 ml of 0–12-h-old embryos essentially according to Prelich *et al.* (1987a). Frozen embryos were thawed and Dounce homogenized in a buffer containing 50 mM Tris-HCl, pH 8, 0.5 mM magnesium acetate, 0.05 mM EDTA, 5 mM KCl, 0.35 M sucrose, 1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride, and 2 μ g/ml leupeptin. The homogenate was filtered through 120- μ m nylon mesh and then spun first at 10,000 \times g and then at 100,000 \times

g. The 100,000 × g (postribosomal) supernatant was loaded onto a phosphocellulose column (600 ml) that had been preequilibrated in buffer A (50 mM Tris-HCl, pH 8, 1 mM EDTA, 0.01% Nonidet P-40, 10% glycerol, 1 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) containing 175 mM NaCl. Protein concentration was determined by the Bio-Rad protein assay. The protein that flowed through the column (phosphocellulose fraction) was loaded onto a DEAE-cellulose column (600 ml) preequilibrated in buffer A with 175 mM NaCl. Proteins were eluted with 1 M NaCl in buffer A. The protein peak (DEAE-cellulose fraction) was adjusted to 1 M (NH₄)₂SO₄ and applied to a phenyl-Sepharose column (45 ml) preequilibrated in buffer A containing 1 M (NH₄)₂SO₄. Proteins were eluted with a linear gradient of decreasing ionic strength formed by buffer A containing 0.5 M (NH₄)₂SO₄ to buffer A alone. PCNA was detected by immunoblotting using monoclonal anti-rabbit PCNA antibodies 19A2 and 19F4. The pooled peak of PCNA was dialyzed against buffer A containing 25 mM NaCl and 20% sucrose. It was then loaded onto a QAE-Sepharose column (11 ml) preequilibrated in buffer A with 0.2 M NaCl. PCNA was eluted with a linear gradient of 0.2–0.6 M NaCl in buffer A. Pooled fractions were dialyzed against 25 mM NaCl and 20% sucrose in buffer A and stored at –70 °C.

Production of Anti-Drosophila PCNA Antiserum—Antibodies to *Drosophila* PCNA were raised in a female New Zealand White rabbit essentially as described by Fisher *et al.* (1982). The QAE-Sepharose fraction prepared from 35 ml of 0–12-h-old embryos was subjected to electrophoresis on a preparative SDS-polyacrylamide (10%) gel. The gel was stained with Coomassie Blue, destained, and dried onto Whatman 3MM paper. The band of PCNA was excised, rehydrated in water, and then homogenized in water with a motor-driven Teflon pestle. PCNA was eluted by shaking the homogenized gel in 100 mM Na₂HPO₄, 0.1% SDS.

Amino Acid Analysis and Sequencing of Drosophila PCNA—PCNA was gel purified and extracted from the sliced gel as described above for preparation of antigen, trichloroacetic acid precipitated, and dissolved in 0.1 M ammonium hydroxide, 0.1% SDS. Amino-terminal protein sequence analysis was performed using an Applied Biosystems 470A sequenator equipped for on-line detection of phenylthiohydantoin derivatives as described (Lees-Miller and Anderson, 1989). Amino acid composition was determined using a Hewlett-Packard Aminoquant system. Before analysis, the protein was hydrolyzed for 24 h at 110 °C in 6 N HCl.

SDS-Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Immunoblot Analysis—SDS-PAGE was essentially according to Laemmli (1970) as modified (Fisher *et al.*, 1982). Proteins were transferred either passively or electrophoretically from SDS-polyacrylamide gels onto nitrocellulose. Immunoblots were probed with specific antibodies (Blake *et al.*, 1984; Smith and Fisher, 1984). Calf alkaline phosphatase was glutaraldehyde conjugated to affinity-purified goat anti-IgG antibodies according to Avrameas (1969); colorimetric detection of alkaline phosphatase activity on blots was according to McGadey (1970).

Indirect Immunofluorescence—Indirect immunofluorescence was performed on *Drosophila* third instar larval tissues exactly as described previously (Fisher *et al.*, 1982). Detection of specific antibody staining was with affinity-purified rhodamine-conjugated donkey anti-rabbit IgG diluted at 1:50. Additional details are included in the figure legends.

In Vitro Replication of Plasmid DNA Containing the SV40 Origin of Replication—Standard replication reactions (25 μl) were performed essentially as described by Tsurimoto *et al.* (1989) and contained plasmid pSV011 as template DNA (Prelich and Stillman, 1988). The reconstituted replication system contained purified SV40 T antigen (Simanis and Lane, 1985), RF-A (Fairman and Stillman, 1988; Wold and Kelly, 1988), RF-C (Tsurimoto and Stillman, 1989a), human PCNA produced from an *Escherichia coli* expression vector² or *Drosophila* PCNA (described herein), DNA topoisomerases I and II, and the nonpurified fraction IIA (Tsurimoto *et al.*, 1989). Reactions were for 1 h at 37 °C, and reaction products were analyzed by trichloroacetic acid precipitation or by electrophoresis on an 0.8% agarose gel as described in the legend to Fig. 4.

RESULTS

Purification of a Putative PCNA Homolog from *Drosophila* Embryo Extracts—Monoclonal antibodies 19A2 and 19F4,

² K. Fien and B. Stillman, unpublished observations.

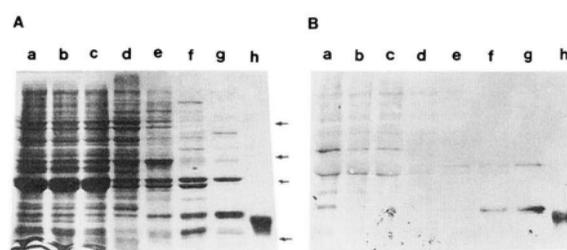


FIG. 1. Purification of *Drosophila* PCNA. A, SDS-PAGE (10%) of fractions generated during purification of *Drosophila* PCNA. Purification and SDS-PAGE were performed as described under "Experimental Procedures." The gel was stained with Coomassie Blue. One unit is defined as the amount of material derived from 1 μl of embryos (~40–50 organisms). Lane a, 0.5 unit of filtered crude homogenate; lane b, 0.5 unit of 100,000 × g supernatant; lane c, 0.5 unit of 100,000 × g supernatant; lane d, 0.5 unit of phosphocellulose flow-through; lane e, 3 units of DEAE-cellulose eluate; lane f, 25 units of phenyl-Sepharose eluate; lane g, 60 units of QAE-Sepharose eluate; lane h, 1 μg of purified human PCNA. B, proteins from a gel run in parallel to A were electrophoretically transferred to nitrocellulose. The resulting blot was probed with monoclonal anti-rabbit PCNA antibodies 19A2 ascites fluid at 1:2000 dilution and 19F4 tissue culture supernatant at 1:30 dilution. Standard molecular mass markers are indicated by arrows to the right of each panel and are (in kDa), from top to bottom: 92.5, 69, 46, and 30.

raised against rabbit PCNA, recognize this protein from both rabbits and humans (Ogata *et al.*, 1987). We considered it possible that these antibodies would also recognize a putative *Drosophila* PCNA homolog. In preliminary experiments, immunoblot analyses performed with these antibodies on *Drosophila* embryo extracts revealed a faint band of immunoreactivity which was apparently specific and migrated at about the same position as human PCNA (not shown). However, also seen were several bands of equal or greater intensity (see Fig. 1B, lane a) which were apparently nonspecific. We therefore set about to purify this minor immunoreactive *Drosophila* protein using a protocol similar to that reported for purification of human PCNA by Prelich *et al.* (1987a). The putative *Drosophila* PCNA homolog was detected during purification by immunoblot analysis using monoclonal antibodies 19A2 and 19F4.

Fig. 1A shows a Coomassie Blue-stained SDS-polyacrylamide gel; Fig. 1B shows an immunoblot of an identical gel, run in parallel to the one shown in Fig. 1A, and probed with a mixture of monoclonal anti-rabbit PCNA antibodies 19A2 and 19F4. Although there appear to be many immunoreactive polypeptides in the crude embryo homogenate (Fig. 1B, lane a), probing parallel blots with only the secondary antibody (not shown) demonstrated that all those seen in Fig. 1B, lane a, were apparently nonspecific. Specific reactivity of an appropriately sized *Drosophila* polypeptide with monoclonal anti-rabbit PCNA antibodies was first evident after DEAE-cellulose chromatography (Fig. 1B, lane e) although this species could be seen in earlier fractions if more material was loaded on the gel.

Two major bands of Coomassie Blue-stainable protein were seen in the QAE-Sepharose eluate (Fig. 1A, lane g). The lower of the two was exactly coincident in SDS-PAGE mobility with the major band of specific reactivity seen on a parallel immunoblot probed with monoclonal antibodies 19A2 and 19F4 (Fig. 1B, lane g). In this analysis, this band migrated slightly slower than a human PCNA standard loaded and run on the same gel (Fig. 1, A and B, lanes h).³ A second band of

³ Before electrophoresis, samples run on gels shown in Figs. 1, 3, and 5 were treated with iodoacetamide. Samples run on the gel shown in Fig. 4A were not. This may account for the slight difference in relative mobilities between *Drosophila* PCNA and human PCNA seen under the two different conditions.

immunoreactivity seen at about 50 kDa in Fig. 1B, lane g, is apparently nonspecific (see Fig. 3D).

Biochemical and Immunochemical Homologies between Drosophila PCNA and Human PCNA—To establish definitively the identity of the major immunoreactive Coomassie Blue-stainable protein band in Fig. 1A, lane g, as a *Drosophila* PCNA homolog, approximately 200 μ g of this polypeptide was purified from the QAE-Sepharose eluate by preparative SDS-PAGE; 26 μ g was subjected to NH₂-terminal sequence analysis, an identical amount was used for determination of total amino acid composition, and the remainder was used to immunize a rabbit for antibody production.

The results of NH₂-terminal sequence analysis are presented in Fig. 2. A single sequence was obtained in the first 10 cycles of Edman degradation, and a major sequence continued and could be read unambiguously for 22 cycles. This sequence was identical to that of human PCNA at 16 of the 22 residues (Fig. 2). The yield of methionine in the 1st residue was 275 pmol, suggesting that in *Drosophila* the initiating methionine residue is not removed from PCNA. Careful inspection of the sequence data (not shown) revealed distinct minor sequences that were closely related to the main sequence beginning at cycles 11, 13, 17, and 19. The minor sequences appeared to result from amino acid insertion, deletion, or substitutions and suggest that PCNA may be encoded by several closely related genes. It is also possible that minor sequences resulted from sequence-specific partial failure of the Edman chemistry causing partial asynchrony of the sequence at certain positions.

The amino acid composition of SDS-PAGE-purified *Drosophila* PCNA is shown in Table I. In comparison with the amino acid composition of human PCNA deduced from a cDNA clone (Almendral *et al.*, 1987), *Drosophila* PCNA is quite similar.

The reactivity of antiserum raised against SDS-PAGE-purified *Drosophila* PCNA was compared by immunoblot analysis with that of monoclonal anti-rabbit PCNA antibodies 19A2 and 19F4. Both human PCNA and the QAE-Sepharose eluate enriched for *Drosophila* PCNA were subjected to electrophoresis on four identical SDS-polyacrylamide gel segments. One segment (Fig. 3A) was stained with Coomassie Blue while proteins from the other three were blot-transferred to nitrocellulose. The blot shown in Fig. 3B was probed with anti-*Drosophila* PCNA antiserum; the blot shown in Fig. 3C was probed with a mixture of monoclonal anti-rabbit PCNA antibodies 19A2 and 19F4; the blot shown in Fig. 3D was probed with secondary antibodies only.

Clearly, the anti-*Drosophila* PCNA antiserum was highly reactive with the antigen against which it was raised (Fig. 3B, lanes b-d). Limited cross-reactivity with human PCNA was also observed (see Fig. 3B, lane e). Conversely, monoclonal



FIG. 2. NH₂-terminal sequence comparison between human PCNA and the putative *Drosophila* homolog. Identical amino acids are connected by dashed lines and are boxed. Amino acids changed conservatively are connected by dashed lines only. Amino acid yields over the first 10 cycles were as follows: cycle 1, 275 pmol of methionine; cycle 2, 242 pmol of phenylalanine; cycle 3, 124 pmol of glutamic acid; cycle 4, 234 pmol of alanine; cycle 5, 37 pmol of arginine; cycle 6, 277 pmol of leucine; cycle 7, 136 pmol of glycine; cycle 8, 132 pmol of glutamine; cycle 9, 189 pmol of alanine; cycle 10, 44 pmol of threonine.

TABLE I
Amino acid composition of *Drosophila* PCNA

Amino acid	nmol	Calculated ^a residues	Human PCNA ^b residues
Lys	7.38	15.8	16
His	1.43	3.1	3
Arg	4.67	10.0	8
Asp + Asn	15.46	33.1	30
Thr	7.43	15.9	12
Ser	7.72	16.4	25
Glu + Gln	15.98	34.1	31
Pro	3.09	6.6	7
Gly	6.21	13.3	14
Ala	11.43	24.4	19
Val	8.75	18.7	21
Met	5.60	12.0	10
Ile	7.26	15.5	14
Leu	11.64	24.9	29
Tyr	2.72	5.8	7
Phe	5.56	11.9	8
Trp			1
Cys ^c			6
Total			261

^a Calculated no. of residues assumes molecular weight = 29,261.

^b Values are derived from the published sequence of human PCNA deduced from a cDNA clone (Almendral *et al.*, 1987).

^c Before SDS-PAGE, the QAE-Sepharose fraction was reduced and treated with iodoacetamide to alkylate —SH groups. Carboxymethylcysteine was identified but not quantitated in the subsequent amino acid analysis.

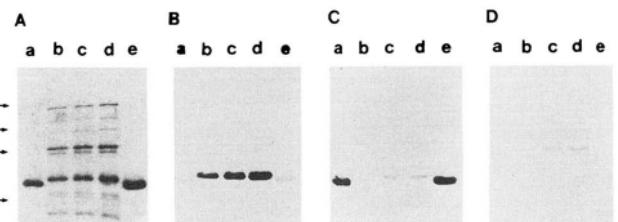


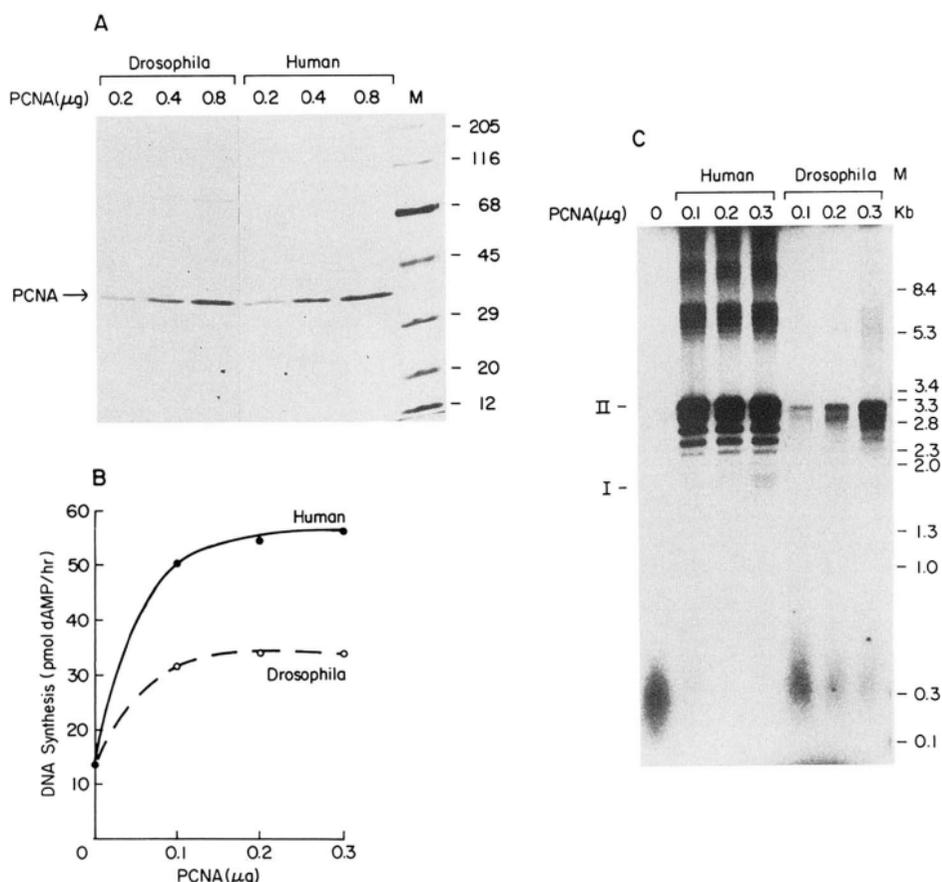
FIG. 3. Immunoblotting homology between *Drosophila* PCNA and human PCNA. *Drosophila* QAE-Sepharose fraction and purified human PCNA were subjected to electrophoresis on SDS-polyacrylamide (10%) gels and proteins transferred electrophoretically to nitrocellulose. QAE-Sepharose fraction was loaded in lane b, 24 units; lane c, 48 units; and lane d, 96 units. Human PCNA was loaded in lane a, 0.25 μ g, and lane e, 0.75 μ g. A, the gel was stained with Coomassie Blue after electrophoresis. Three immunoblots (B-D) were prepared from gels run in parallel. The blot shown in B was probed with anti-*Drosophila* PCNA antiserum at 1:1000 dilution. The blot shown in C was probed with monoclonal anti-rabbit PCNA antibodies 19A2 ascites fluid, diluted at 1:2000 and 19F4 tissue culture supernatant, diluted at 1:30. The blot shown in D was probed with calf alkaline phosphatase-conjugated goat anti-mouse and goat anti-rabbit IgG antibodies only. Arrows to the left of panel A designate marker positions as in Fig. 1.

anti-rabbit PCNA antibodies were highly cross-reactive with human PCNA (Fig. 3C, lanes a and e) and showed only limited cross-reactivity with *Drosophila* PCNA (Fig. 3C, lanes b-d).

Functional Homology between *Drosophila* PCNA and Human PCNA—In light of the high degree of conservation between *Drosophila* PCNA and human PCNA, we thought it possible that *Drosophila* PCNA might substitute for human PCNA in the reconstituted SV40 *in vitro* DNA replication system. *Drosophila* PCNA was purified for this experiment under conditions to maximize purity. This entailed taking very narrow pools of PCNA elution peaks in each of the last two column chromatography steps.

The purified PCNA fractions from both *Drosophila* and humans were compared by SDS-PAGE (Fig. 4A). In this analysis, the two proteins migrated nearly identically.³ The

FIG. 4. Functional homology between *Drosophila* PCNA and human PCNA. A, SDS-PAGE analysis of purified *Drosophila* PCNA and human PCNA. Following electrophoresis, the gel was stained with Coomassie Blue to show the amounts of *Drosophila* PCNA and human PCNA added to each reaction. B, stimulation of *in vitro* SV40 DNA replication by *Drosophila* and human PCNA. Reactions, 25 μ l each, contained 150 ng of pSV011 plasmid DNA, 0.5 μ g of SV40 T antigen, 200 ng of DNA topoisomerase I, 90 ng of DNA topoisomerase II, 650 ng of RF-A, 100 ng of RF-C, 20 μ g of fraction IIA, and increasing amounts of PCNA as indicated. Reactions were incubated at 37 $^{\circ}$ C for 1 h. A portion of each reaction was terminated by the addition of EDTA to 10 mM, precipitated by 8% trichloroacetic acid containing 1% sodium pyrophosphate, and filtered through glass fiber filters. Amounts of incorporated [32 P] dAMP were determined by liquid scintillation counting. C, the other portions of replication products were analyzed by agarose gel electrophoresis. Positions of form I and II products are indicated on the left.



effects of adding either to an otherwise complete SV40 DNA replication reaction were determined both quantitatively and qualitatively. From the quantitative analysis (Fig. 4B), it can be seen that both proteins stimulated the rate of dAMP incorporation with similar kinetics. However, the maximal human PCNA-stimulated rate was about twice that observed for *Drosophila* PCNA. From the qualitative analysis (Fig. 4C), it was clear that *Drosophila* PCNA acted in a manner that was mechanistically homologous to human PCNA, *i.e.* it facilitated the synthesis of full-length SV40 DNA whereas in its absence, short non-template-bound products were observed which are similar to those described previously (Prelich and Stillman, 1988). These short products are variably lost during drying of the agarose gel prior to autoradiography, perhaps accounting for the apparent discrepancy in saturation kinetics between the dAMP incorporation assay (Fig. 4B) and product analysis on agarose gels (Fig. 4C). The product analysis rather than measurement of acid insoluble radioactivity more accurately reflects *Drosophila* PCNA function.

Steady-state Levels of *Drosophila* PCNA during Development—Polyclonal anti-*Drosophila* PCNA antibodies were used for immunoblot analyses to evaluate the relative steady-state levels of PCNA through development. During embryogenesis, PCNA levels were highest early when rates of DNA replication were maximal and decreased markedly as development proceeded (Fig. 5A). In contrast, steady-state levels of two other nuclear proteins, DNA topoisomerase II (Fig. 5B) and lamin (Fig. 5C), seemed to increase throughout the same period. Also evident on the blot probed with anti-PCNA antiserum in Fig. 5A is an immunoreactive polypeptide of approximately 50 kDa which seems to be regulated in a manner similar to PCNA during embryogenesis. Reactivity of the anti-*Drosophila* PCNA antiserum with this 50-kDa poly-

peptide in crude extracts is apparently specific (compare Fig. 5A with Fig. 5B; also see below).

Results of immunoblot analysis of later developmental stages with anti-*Drosophila* PCNA antibodies are presented in Fig. 5D. Only the region of the blot containing PCNA is shown. Most notable in this experiment were the increased levels of PCNA seen in adult females as opposed to males (compare Fig. 5D, lanes *i* and *j*). This finding is consistent with the notion that the elevated levels of PCNA early in embryogenesis represent maternal stores of this protein accumulated during oogenesis.

In Situ Immunolocalization of *Drosophila* PCNA—Developmental immunoblot studies with anti-*Drosophila* PCNA antibodies were complemented by indirect immunofluorescence analyses performed on selected tissues dissected from *Drosophila* third instar larvae. For these experiments, anti-PCNA antibodies were affinity purified on a PCNA-Sepharose column (see "Experimental Procedures"). Although affinity purification reduced the level of apparently nonspecific background seen both on immunoblots and by immunofluorescence, blot immunoreactivity with the 50-kDa species seen with unfractionated serum (Fig. 5A) was undiminished with affinity-purified anti-PCNA IgG (not shown). Therefore, in considering immunofluorescence data, although we think it unlikely, we cannot exclude the possibility that patterns observed reflect the distribution of this 50-kDa protein in addition to or instead of those of PCNA.

The third instar larval neural ganglion is a heterogeneous tissue made up of many different cell types. Most are small cells that are apparently nondividing, but some of the cells are much larger, and mitotic figures are easily found among this group. The distribution of PCNA in third instar larval neural ganglion tissue was evaluated by indirect immunoflu-

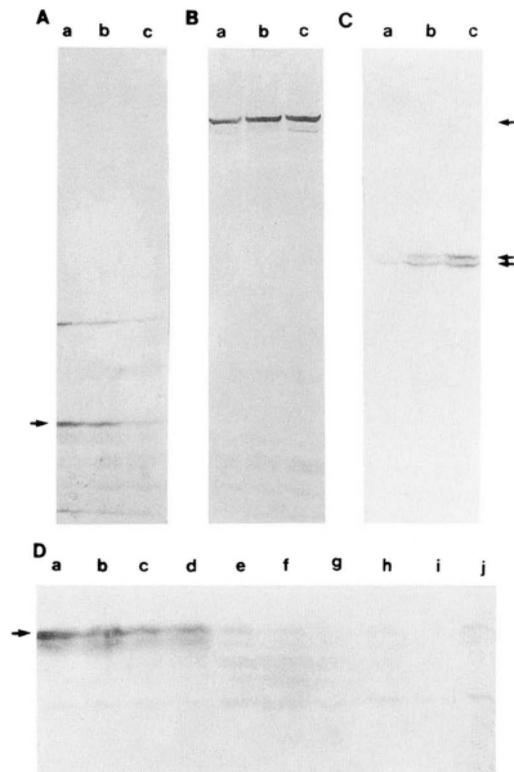


FIG. 5. Immunoblot analysis of *Drosophila* PCNA levels through development. Electrophoresis was on SDS-polyacrylamide (7%) gels. Proteins were transferred passively to nitrocellulose, and blots were processed and probed as described under "Experimental Procedures." A-C, total extracts from embryos of various ages were prepared by Dounce homogenization of dechorionated embryos directly into boiling SDS; 500 μ g of protein from each extract was loaded onto each of three parallel gel segments; lanes a, 0-3-h-old embryos; lanes b, 6-9-h-old embryos; lanes c, 12-15-h-old embryos. The blot in panel A was probed with anti-*Drosophila* PCNA antiserum diluted 1:1000; similar results were obtained with affinity-purified anti-*Drosophila* PCNA IgG (not shown). The blot in panel B was probed with anti-*Drosophila* topoisomerase II antiserum diluted 1:1000. The blot in panel C was probed with affinity-purified anti-*Drosophila* lamin IgG at a final concentration of 0.5 μ g/ml. D, extracts were prepared and processed as in A-C but from *Drosophila* at all developmental stages; lane a, 0-3-h-old embryos; lane b, 6-9-h-old embryos; lane c, 12-15-h-old embryos; lane d, 19-22-h-old embryos; lane e, first instar larvae; lane f, second instar larvae; lane g, third instar larvae; lane h, pupae; lane i, adult males; lane j, adult females. The blot was probed with anti-*Drosophila* PCNA antiserum as in A. Only a portion of the blot is shown. Arrows to the left of panels A and D indicate the mobility of a purified *Drosophila* PCNA standard run in parallel to the samples used for immunoblot analysis. Arrows to the right of panel C indicate the mobility of *Drosophila* DNA topoisomerase II (upper arrow), 166 kDa; and *Drosophila* nuclear lamins (lower doublet of arrows) Dm₂ (76 kDa) and Dm₁ (74 kDa).

orescence microscopy. The results of this analysis are shown in Fig. 6. Fig. 6, a, d, and g are phase-contrast micrographs; Fig. 6, b, e, and h are immunofluorescence micrographs; Fig. 6, c, f, and i are fluorescence micrographs of the same cells stained with the DNA-specific dye DAPI. The specimens in panels a-c and d-f were probed with affinity-purified anti-PCNA antibodies; the specimen in panels g-i was probed with preimmune rabbit IgG.

From the specimens in Fig. 6, a-c and d-f, it can be seen that most or all of the small cells in the field, as revealed by DAPI staining of their nuclei (Fig. 6, c and f), showed little or no specific staining with anti-PCNA antibodies (Fig. 6, b and e). In contrast, all of the large cells in Fig. 6, a-c, stained relatively intensely. Careful comparison of phase-contrast

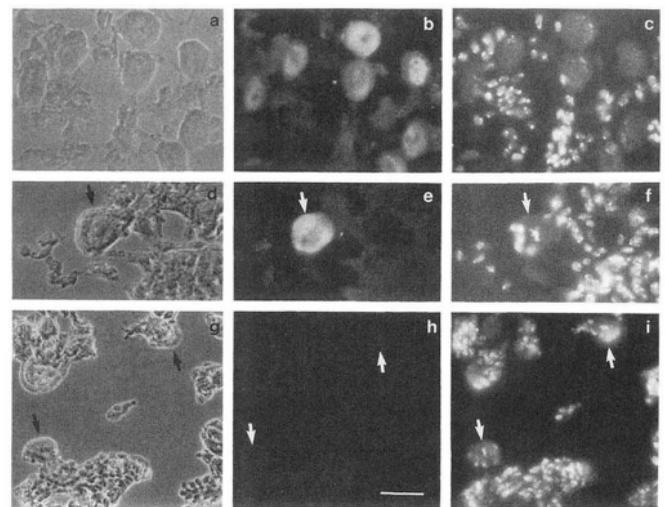


FIG. 6. Immunolocalization of *Drosophila* PCNA in interphase and mitotic cells. Neural ganglia were prepared for indirect immunofluorescence and processed as described under "Experimental Procedures." Specimens in a-f were probed with affinity-purified anti-*Drosophila* PCNA IgG at a final concentration of 0.125 μ g/ml; specimens in g-i were probed with preimmune IgG at a final concentration of 1 μ g/ml. Phase-contrast (a, d, and g), immunofluorescence (b, e, and h), and DAPI fluorescence (c, f, and i) micrographs are shown. Arrows indicate mitotic cells. The bar in panel h designates 20 μ m and applies to all panels.

(Fig. 6a), immunofluorescence (Fig. 6b), and DAPI fluorescence micrographs (Fig. 6c) revealed that in most of these large cells, PCNA was apparently distributed diffusely throughout the nucleus with the possible exception of the nucleolus. In Fig. 6, d-f, a mitotic cell is shown; it stained intensely throughout with anti-PCNA antibodies. All other cells in this field were negative.

Polytene chromosomes are found in many different *Drosophila* cells and tissues. The process of polytenization is thought to involve ongoing DNA replication without separation of daughter DNA molecules between successive rounds. This results in a polytene chromosome, easily visible at the light microscopic level, with as many as 1,000 copies of duplex DNA aligned linearly. The presence of PCNA in *Drosophila* polytene chromosomes was evaluated by indirect immunofluorescence microscopy using squash preparations of third instar larval salivary gland giant cells. Results of this analysis are shown in Fig. 7. Fig. 7, a and c, are phase-contrast micrographs; Fig. 7, b and d, are immunofluorescence micrographs. The specimen in Fig. 7, a and b, was probed with affinity-purified anti-PCNA antibodies; the specimen in Fig. 7, c and d, was probed with preimmune rabbit IgG. Clearly, there is specific staining of the polytene chromosomes along their entire lengths, with obvious heterogeneity in the pattern. This heterogeneity is of uncertain significance.

DISCUSSION

In this paper, we report the identification and purification of a PCNA homolog from *D. melanogaster* embryos. NH₂-terminal amino acid sequence analysis as well as determinations of total amino acid composition indicate a high degree of conservation between *Drosophila* PCNA and mammalian PCNA; functional studies demonstrate that *Drosophila* PCNA can substitute for human PCNA in the reconstituted SV40 DNA replication reaction. These observations, in conjunction with results of developmental and immunocytochemical analyses, suggest that in *Drosophila*, PCNA plays a role in DNA

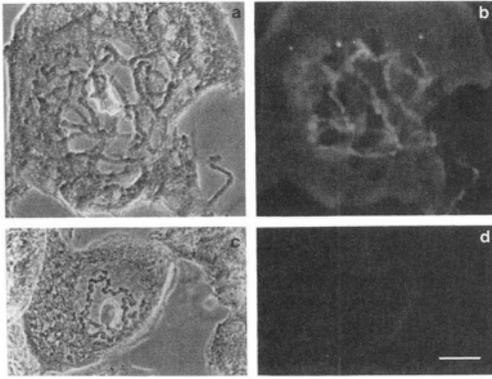


FIG. 7. Polytene chromosome staining with anti-*Drosophila* PCNA IgG. Salivary glands of third instar larvae were prepared and processed as described under "Experimental Procedures." The specimen in *a* and *b* was probed with affinity-purified anti-*Drosophila* PCNA IgG at a final concentration of 0.125 μ g/ml; the specimen in *c* and *d* was probed with preimmune IgG at a final concentration of 1 μ g/ml. Phase-contrast (*a* and *c*) and immunofluorescence micrographs (*b* and *d*) are shown. The bar in panel *d* designates 20 μ m and applies to all panels.

replication similar to that proposed for mammalian and yeast cells.

In mammals, PCNA acts as a processivity factor for DNA polymerase δ . To date, DNA polymerase δ has not been identified in *Drosophila*. The identification of a *Drosophila* PCNA homolog suggests that a DNA polymerase δ homolog exists as well. Results of preliminary experiments performed in one of our laboratories indicate that there is indeed a DNA polymerase activity in *Drosophila* embryo extracts which is stimulated by PCNA and can be distinguished from DNA polymerase α immunochemically.⁴

The postulated role of mammalian PCNA to promote the processivity of DNA polymerase δ and to facilitate coordinated leading and lagging strand replication by DNA polymerases δ and α , respectively, suggests that PCNA interacts with DNA polymerase δ as well as with other proteins in the replication fork. Recently, human PCNA was shown to interact with another replication factor, RF-C; RF-C stimulates both polymerases α and δ (Tsurimoto and Stillman, 1989b, 1990). That *Drosophila* PCNA can substitute for human PCNA in the reconstituted SV40 DNA replication system suggests that those PCNA domains important for interactions with both DNA polymerase δ and RF-C are conserved between *Drosophila* and humans. In contrast, yeast PCNA can stimulate mammalian DNA polymerase δ (Bauer and Burgers, 1988) but cannot replace human PCNA during SV40 DNA replication *in vitro*.⁵ Comparison of complete PCNA sequences from all three species once they are available may therefore yield important insights into the organization of functional domains on the protein.

In mammalian cells, the synthesis of PCNA is partially regulated during the cell cycle, with the highest rates being observed in late G₁ and early S phase (see Bravo and Macdonald-Bravo, 1985; Morris and Matthews, 1989). More striking, however, are the changes in the immunofluorescent staining pattern observed with anti-PCNA antibodies (Celis and Celis, 1985a; Bravo and Macdonald-Bravo, 1985, 1987). During S phase, PCNA is readily detectable, and intranuclear distribution of PCNA changes both qualitatively and quantitatively as DNA replication progresses. During other phases of the cell cycle, with the possible exception of mitosis (Bravo

and Macdonald-Bravo, 1987), PCNA is virtually undetectable by immunocytochemical techniques following methanol fixation of cells, this despite the fact that immunoblot analyses demonstrate the continued presence of this protein (Wold *et al.*, 1988; Morris and Matthews, 1989). In contrast, in fully differentiated nondividing tissues, PCNA is undetectable either by immunoblot or immunofluorescence analysis.

Results of developmental immunoblot analyses in *Drosophila* are consistent with observations made in mammalian cells. The decrease in absolute levels of PCNA during embryogenesis, in contrast to the behavior of two other nuclear proteins, lamin and DNA topoisomerase II, suggests that as cells stop replicating and dividing during embryogenesis, new synthesis of PCNA slows (stops), and eventually the protein is lost. At later developmental stages, there is relatively little DNA synthesis that occurs, and this is reflected by the relative lack of PCNA. The most notable exception to this trend can be appreciated by comparing adult males with adult females. Although undetectable in the former, a band with the expected SDS-PAGE mobility of PCNA is seen in the latter. In light of the high levels of PCNA in early embryos, we think it likely that PCNA detectable in adult females is derived from the ovary where it is being stockpiled in the developing oocyte along with other proteins needed in large quantities early in embryogenesis (see *e.g.* Smith and Fisher, 1989). This suggestion will need to be evaluated by *in situ* immunocytochemistry.

The results of developmental immunoblot analyses were corroborated by those of indirect immunofluorescence experiments performed with affinity-purified anti-PCNA antibodies. In cryosections through third instar larvae where PCNA was virtually undetectable on immunoblots, little immunofluorescent staining was demonstrable overall.⁶ However, when selected third instar larval tissues known to contain cells active in DNA replication were examined, PCNA could be identified. In neural ganglion tissue, although we cannot be certain that those cells that were positive for PCNA were actually replicating their DNA, the number of cells in which staining was observed was roughly what might be predicted based on the mitotic index of the tissue. In this context, it is noteworthy that all mitotic cells examined stained intensely with anti-PCNA antibodies, in contrast to mammalian tissue culture in which mitotic cells stain very weakly compared with S phase cells. In salivary gland cells, staining of polytene chromosomes was readily demonstrable in all nuclei examined, provided that young third instar larvae were used. Polytenization occurs through DNA replication. The presence of PCNA in polytene chromosomes suggests that both DNA polymerases α and δ are involved.

In conclusion, the identification of PCNA in *Drosophila*, the availability of polyclonal anti-PCNA antibodies, and the fact that these antibodies are apparently useful probes for the presence of PCNA both on immunoblots and *in situ* should facilitate detailed analysis of this protein and patterns of cell division during differentiation and development. Results of such studies are likely to provide novel insights into the role of cell proliferation in regulating these processes.

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REFERENCES

- Allis, C. D., Waring, G. L., and Mahowald, A. P. (1977) *Dev. Biol.* **56**, 372-381
 Almendral, J. M., Huebsch, D., Blundell, P. A., Macdonald-Bravo,

⁴ L. Ng, unpublished observations.

⁵ G. Prelich and B. Stillman, unpublished observations.

⁶ P. Fisher, unpublished observations.

- H., and Bravo, R. (1987) *Proc. Natl. Acad. Sci. U. S. A.* **84**, 1575-1579
- Avrameas, S. (1969) *Immunochemistry* **6**, 43-52
- Bauer, G. A., and Burgers, P. M. J. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 7506-7510
- Berrios, M., Osheroff, N., and Fisher, P. A. (1985) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 4142-4146
- Blake, M. S., Johnston, K. H., Russell-Jones, G. J., and Gotschlich, E. C. (1984) *Anal. Biochem.* **136**, 175-179
- Bravo, R., and Celis, J. E. (1980) *J. Cell Biol.* **84**, 795-802
- Bravo, R., and Macdonald-Bravo, H. (1985) *EMBO J.* **4**, 655-661
- Bravo, R., and Macdonald-Bravo, H. (1987) *J. Cell Biol.* **105**, 1549-1554
- Bravo, R., Fey, S. J., Bellatin, J., Larsen, P. M., Arevalo, J., and Celis, J. E. (1981) *Exp. Cell Res.* **136**, 311-319
- Bravo, R., Frank, R., Blundell, P. A., and Macdonald-Bravo, H. (1987) *Nature* **326**, 515-517
- Celis, J. E., and Celis, A. (1985a) *Proc. Natl. Acad. Sci. U. S. A.* **82**, 3262-3266
- Celis, J. E., and Celis, A. (1985b) *EMBO J.* **4**, 1187-1192
- Celis, J. E., Madsen, P., Celis, A., Nielsen, H. V., and Gesser, B. (1987) *FEBS Lett.* **220**, 1-7
- Fairman, M. P., and Stillman, B. (1988) *EMBO J.* **7**, 1211-1218
- Fisher, P. A., and Smith, D. E. (1988) *Biochem. Soc. Trans.* **16**, 134-138
- Fisher, P. A., Berrios, M., and Blobel, G. (1982) *J. Cell Biol.* **92**, 674-686
- Laemmli, U. K. (1970) *Nature* **227**, 680-685
- Lees-Miller, S. P., and Anderson, C. W. (1989) *J. Biol. Chem.* **264**, 2431-2437
- Mathews, M. B., Bernstein, R. M., Franza, B. R., Jr., and Garrels, J. I. (1984) *Nature* **309**, 374-376
- McGadey, J. (1970) *Histochemie* **23**, 180-184
- Miyachi, K., Fritzler, M. J., and Tan, E. M. (1978) *J. Immunol.* **121**, 2228-2234
- Morris, G. F., and Mathews, M. B. (1989) *J. Biol. Chem.* **264**, 13856-13864
- Ogata, K., Kurki, P., Celis, J. E., Nakamura, R. M., and Tan, E. M. (1987) *Exp. Cell Res.* **168**, 475-486
- Olins, D. E., Olins, A. L., Cacheiro, L. H., and Tan, E. M. (1989) *J. Cell Biol.* **109**, 1399-1410
- Prelich, G., and Stillman, B. (1988) *Cell* **53**, 117-126
- Prelich, G., Kostura, M., Marshak, D. R., Mathews, M. B., and Stillman, B. (1987a) *Nature* **326**, 471-475
- Prelich, G., Tan, C.-K., Kostura, M., Mathews, M. B., So, A. G., Downey, K. M., and Stillman, B. (1987b) *Nature* **326**, 517-520
- Simanis, V., and Lane, D. P. (1985) *Virology* **144**, 88-100
- Smith, D. E., and Fisher, P. A. (1984) *J. Cell Biol.* **99**, 20-28
- Smith, D. E., and Fisher, P. A. (1989) *J. Cell Biol.* **108**, 255-265
- Smith, D. E., Gruenbaum, Y., Berrios, M., and Fisher, P. A. (1987) *J. Cell Biol.* **105**, 771-790
- Tan, C.-K., Castillo, C., So, A. G., and Downey, K. M. (1986) *J. Biol. Chem.* **261**, 12310-12316
- Tan, E. M. (1989) *Adv. Immunol.* **44**, 93-151
- Tsurimoto, T., and Stillman, B. (1989a) *Mol. Cell Biol.* **9**, 609-619
- Tsurimoto, T., and Stillman, B. (1989b) *EMBO J.* **8**, 3883-3889
- Tsurimoto, T., and Stillman, B. (1990) *Proc. Natl. Acad. Sci. U. S. A.* **87**, 1023-1027
- Tsurimoto, T., Fairman, M. P., and Stillman, B. (1989) *Mol. Cell Biol.* **9**, 3839-3849
- Wold, M. S., and Kelly, T. (1988) *Proc. Natl. Acad. Sci. U. S. A.* **85**, 2523-2527
- Wold, M. S., Li, J. J., Weinberg, D. H., Virshup, D. M., Sherley, J. L., Verheyen, E., and Kelly, T. (1988) in *Eukaryotic DNA Replication in Cancer Cells* (Kelly, T., and Stillman, B., eds) Vol. 6, pp. 133-141, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
- Zuber, M., Tan, E. M., and Ryoji, M. (1989) *Mol. Cell Biol.* **9**, 57-66