

Role of Homeodomain Protein Binding Region in the Expression of *Drosophila* Proliferating Cell Nuclear Antigen Gene: Analysis With Transgenic Flies

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The regulatory region of *Drosophila* proliferating cell nuclear antigen (PCNA) gene consists of a promoter region (−168 to +24 with respect to the transcription initiation site) and an upstream region containing three homeodomain protein binding sites (HDB) (−357 to −165). The PCNA gene regulatory regions with HDB (−607 to +137) or without HDB (−168 to +137) were fused with the *lacZ* and transgenic flies were established by P-element-mediated transformation. Male transgenic flies were crossed with wild-type females, and zygotic expression of the *lacZ* was monitored by quantitative β-galactosidase assay, at various stages of development. Expression of the *lacZ* was high in embryos, first and second instar larvae, and adult females, and low at other stages of development. Only a marginal difference in expression was observed between flies carrying the homeodomain protein binding region and those not carrying it. Spatial pattern of the *lacZ* expression in the embryo visualized by immunostaining with the anti-*lacZ* antibody was similar to the distribution of the endogenous PCNA protein. Here, too, only a marginal difference was observed between transgenic flies carrying two different constructs of the PCNA *lacZ*. In genetic crossing experiments of transgenic flies with those carrying mutation in homeobox genes, no significant change in the *lacZ* expression pattern was observed. However, when male transgenic flies were crossed with female flies homozygous for a *torso* gain-of-function allele, repression of the *lacZ* expression was observed in the central region of the embryo. Because these local changes in the *lacZ* expression depend on the homeodomain protein binding region, unidentified homeodomain proteins are probably involved. Our results suggest that the promoter region is practically sufficient for expression of the PCNA gene and that the homeodomain protein binding region functions as a silencer when *torso* is activated ectopically.

Homeodomain protein binding region *Drosophila* proliferating cell nuclear antigen
Transgenic flies

THE proliferating cell nuclear antigen (PCNA), known as an accessory protein of DNA polymerase δ, is required for simian virus 40 DNA replication as well as for cellular DNA replication (Tsurimoto et al., 1990; Jaskulski et al., 1988; Liu et

al., 1989). PCNA is also involved in DNA repair (Shivji et al., 1992). The amino acid sequence of the PCNA protein has been highly conserved in a wide range of species from yeast to human (reviewed by Moriuchi, 1990).

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We earlier cloned and sequenced the *Drosophila* PCNA gene and its 5'-flanking region (Yamaguchi et al., 1990). The presence of clusters of 10-bp sequences, similar to the binding consensus for *Drosophila* homeodomain proteins (Desplan et al., 1988; Hoey and Levine, 1988; Muller et al., 1988), was noted in the region from nucleotide positions -165 to -357 of the *Drosophila* PCNA gene. DNase I footprint analysis revealed that the homeodomain proteins encoded by a pair rule gene, *even-skipped* (*eve*), and a dorso-ventral gene, *zerknüllt* (*zen*), can specifically bind to these sequences in vitro (Yamaguchi et al., 1990). Studies in vivo and in cultured cells revealed that *zen* could repress expression of the PCNA gene (Yamaguchi et al., 1991b). However, the repression appeared to be indirect because the target region of *zen* for the repression was mapped in the promoter region of the PCNA gene to which *zen* did not bind in vitro (Yamaguchi et al., 1991b; Hirose et al., 1994). Therefore, it remained to be determined whether or not the homeodomain protein binding region of the PCNA gene truly functions in vivo.

In the present study, we used transgenic flies carrying the PCNA gene regulatory regions fused with the *lacZ* (Yamaguchi et al., 1991b) to examine effects of the homeodomain protein binding region on the PCNA gene promoter function in vivo. We found that the region containing homeodomain protein binding sites exerts only a marginal effect on the promoter during normal developmental processes, but has the potential to modulate the promoter locally, because spatial change of the *lacZ* expression that depended on the homeodomain protein binding region was observed in *torso* mutant embryos.

MATERIALS AND METHODS

Establishment of Transgenic Flies

The plasmid p5'-607DPCNA*lacZ*W8HS (Yamaguchi et al., 1991b) contains the PCNA gene fragment spanning from -607 to +137 fused with the *lacZ* in a P-element vector. The plasmid p5'-168DPCNA*lacZ*W8HS (Yamaguchi et al., 1991b) contains the PCNA gene fragment spanning from -168 to +137 fused with the *lacZ* in a P-element vector. By P-element-mediated transformation as described by Robertson et al. (1988), 25 and 13 independent transformant lines were established for the p5'-607DPCNA*lacZ*W8HS and the p5'-168DPCNA*lacZ*W8HS, respectively.

These transgenic fly strains and their chromosomal linkages are listed in Table 1.

Analysis of Expression Patterns for PCNA lacZ and Endogenous PCNA

Quantitative measurement of β -galactosidase activity in extracts (Friedell and Searles, 1992). Male transgenic flies were crossed with female wild-type (Canton S) flies. Fifty to 100 individuals each of dechorionated embryos, larvae, pupae, and adult flies were homogenized in 500 μ l of ice-cold assay buffer (50 mM potassium phosphate, pH 7.5/1 mM MgCl₂). Homogenates were centrifuged at 10,000 \times g at 4°C for 5 min. For each assay, 50–200 μ l of the supernatant was added to

TABLE 1
THIRTY-EIGHT TRANSFORMANTS CARRYING THE
lacZ GENE FUSED TO THE
PCNA GENE 5'-FLANKING SEQUENCES

P-Element Plasmids	Strains	Chromosome Linkage
p5'-607DPCNA <i>lacZ</i> W8HS	52	II
	57	X
	60	X
	67	III
	88	II
	95A	III
	95B	II
	96	II
	109	X
	128	II
	133	III
	143A	III
	143B	III
	158	III
	164	III
	183	II
	186	X
	208	III
	218	X
	237	II
244A	III	
244B	III	
248A	III	
258	II	
p5'-168DPCNA <i>lacZ</i> W8HS	5	II
	10	III
	19A	III
	19B	II
	21	X
	22	III
	24A	II
	24B	X
	70	II
	72	II
84	II	
89	II	
98	II	

give 1 ml of assay buffer containing 1 mM chlorophenol red- β -D-galactopyranoside substrate (CPRG; Boehringer Mannheim). Reactions were incubated at 37°C in the dark. The substrate conversion was measured at 574 nm using a spectrophotometer at 0.25, 0.5, 0.75, 1, and 1.5 h after addition of the extract; the rate of color development was linear. The β -galactosidase activity was defined as absorbance units per hour per milligram of protein. To correct for endogenous β -galactosidase activity, extracts from the wild-type strain were included in each experiment and this background reading was subtracted from readings obtained with each transformant line. The protein concentration of the extracts was determined by the Bio-Rad protein assay.

Immunocytochemical method. Male transgenic flies were crossed with female *white (w)* flies to observe zygotic expression of the PCNA *lacZ* in early embryos (0–6 h old). Female transgenic flies were crossed with male *w* flies to observe the maternal contribution to the PCNA *lacZ* expression. To test for expression of the PCNA *lacZ* fusion gene in embryos carrying a mutation in *Antennapedia (Antp)*, mapped on the third chromosome, males heterozygous for the *Antp* mutation (*Antp*^{w10}/TM3) with the transgene on the X chromosome were crossed with *Antp*^{w10} (Lindsley and Zimm, 1992) heterozygous females. Mutations in *Deformed (Dfd)*, *fushi tarazu (ftz)*, *proboscipedia (pb)*, *Sex combs reduced (Scr*^{7F28}*)*, and *Abdominal B (AbdB*^{M1}*, AbdB*^{M5}*, and AbdB*^{rx23-1}*)* are all mapped on the third chromosome (Lindsley and Zimm, 1992). Accordingly, genetic crossing experiments were conducted in the same manner. To test for expression of the PCNA *lacZ* in homozygous mutants of *tor*, male transgenic flies were crossed with female flies homozygous for the *tor*^{PM} (Casanova and Struhl, 1989) or the *tor*^{RL3} (Casanova and Struhl, 1989) mutation. Because *tor*^{RL3} was a ts-mutant, crossing with the *tor*^{RL3} was conducted at 28°C.

Expression patterns of the PCNA *lacZ* and the endogenous PCNA were analyzed by an immunohistochemical method, as described previously (Yamaguchi et al., 1991b). Embryos 0–6 h old were collected, dechorionated, fixed, and devitelinized. Embryos were incubated with the primary antibody followed by treatment with the secondary antibody, as described previously (Yamaguchi et al., 1991b). A polyclonal rabbit anti-*Drosophila* PCNA (Ng et al., 1990) (used at a dilution of 1 : 2000) and an alkaline phosphatase-conjugated goat anti-rabbit IgG (Promega-Biotec,

preabsorbed against 0–16-h-old fixed embryos and used at a dilution of 1 : 2000) served as the primary and secondary antibodies for detection of PCNA. A monoclonal mouse anti-*lacZ* (Promega-Biotec, preabsorbed against 0–16-h-old fixed embryos and used at a dilution of 1 : 1000) and an alkaline phosphatase-conjugated goat anti-mouse IgG (Promega-Biotec, preabsorbed against 0–16-h-old fixed embryos and used at a dilution of 1 : 2000) served as primary and secondary antibodies, respectively, for detection of PCNA *lacZ*. Histochemical color reaction for alkaline phosphatase was done as described previously (Yamaguchi et al., 1991b). Samples were examined under a microscope (Nikon FXA-A1) and photographed on TMAX 100 films (Kodak).

RESULTS

Distribution of PCNA Protein During Early Embryogenesis

Distribution of PCNA protein during embryonic development was analyzed by staining whole-mount embryos with a polyclonal anti-*Drosophila* PCNA antibody (Ng et al., 1990). Specificity of the antibody was verified by immunoblotting analysis (Ng et al., 1990; Yamaguchi et al., 1991a). This antibody specifically reacted with the PCNA protein in crude extracts of 0–3-h-old embryos and with that produced in *E. coli* carrying the expression plasmid for *Drosophila* PCNA (Yamaguchi et al., 1991a).

Strong staining of nuclei above the weak staining of ooplasm was detected throughout 13 nuclear division cycles (Fig. 1A) and at the cellular blastoderm stage (Fig. 1B). At the onset of gastrulation, strong staining was observed in apparently mesodermal cells within the ventral furrow and staining in the nuclei of most of the surface cells remained visible (Fig. 1C,D). During the early phase of germ band extension (stage 7), strong staining of the PCNA was seen in regions of the cephalic furrow, the amnioproctodeal invagination, the anterior midgut primordium, and the invaginated mesoderm (Fig. 1E,F), although weak staining in ectodermal cell nuclei was visible. No significant staining was detected in the dorsal region corresponding to the amnioserosa at this and later stages. During the germ band extension stage, anti-PCNA staining was observed throughout the germ band with stronger staining in the mesodermal layer, as well as within the anterior midgut primordium, the posterior midgut primordium, and the proctodeal primordium (Fig. 1G–

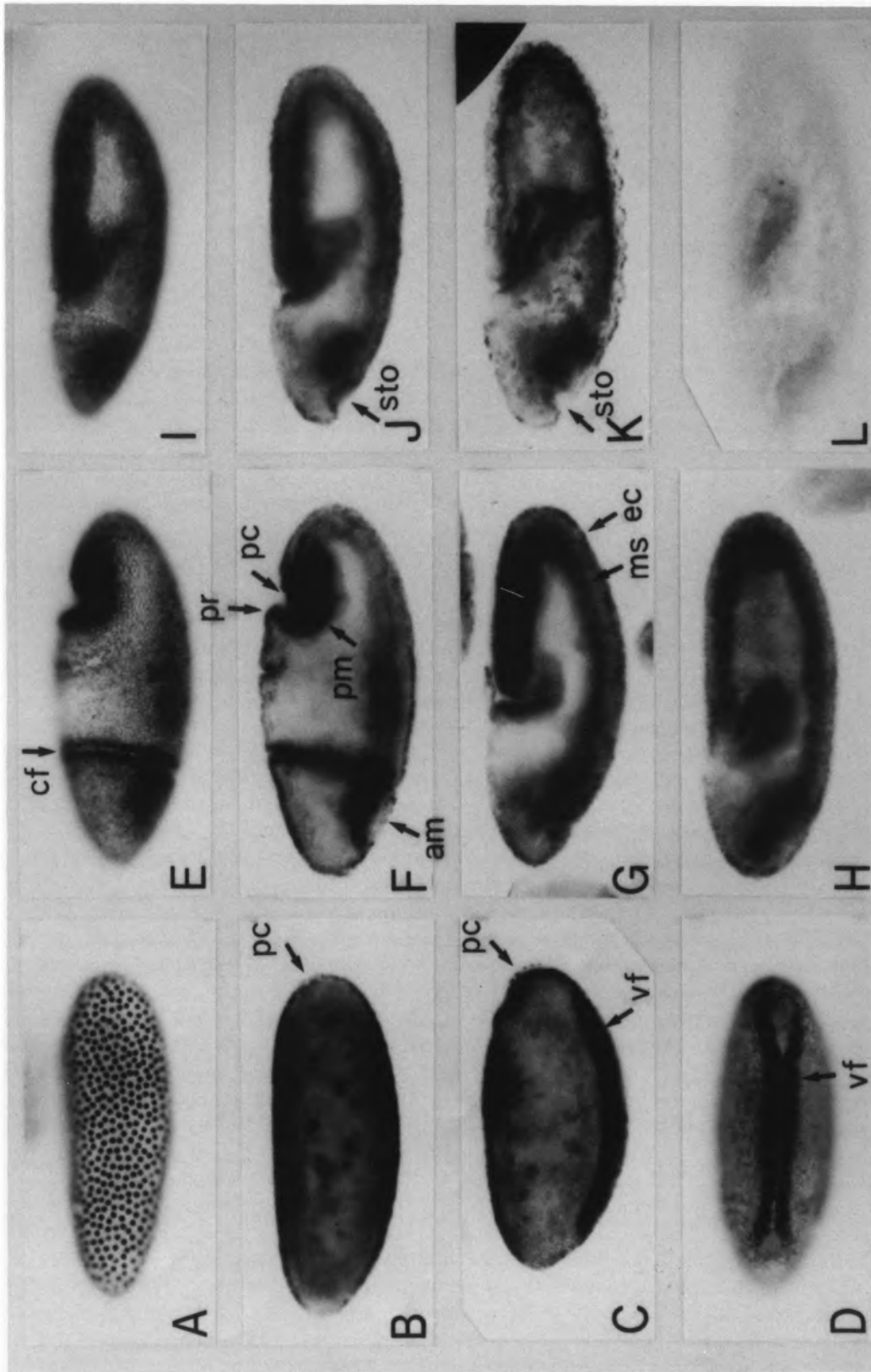


FIG. 1. Distribution of PCNA protein in wild-type embryos. Whole-mount specimens of fixed embryos were immunostained with an anti-PCNA antibody. Embryos are oriented so that anterior is to the left and ventrals are to the bottom [except in (D)]. Staging is according to Campos-Ortega and Hartenstein (1985). (A) Surface view of the syncytial blastoderm embryo (stage 4, 1320–1410 h). (B) Internal focal view of the blastoderm embryo (stage 5, 1410–1450 h), pole cells (pc). (C) Internal focal view of the early gastrulating embryo (stage 6, 1450–1500 h), ventral furrow (vf). (D) Ventral surface view of the early gastrulating embryo (stage 6). (E) Surface view of the embryo at the stage of the beginning of germ band extension (stage 7, 1500–1510 h), cephalic furrow (cf), anterior midgut primordium (am), proctodaeum primordium (pr), posterior midgut primordium (pm). (G) Internal focal view of the stage 8 embryo (1510–1540 h) (germ band elongation), mesoderm (ms), ectoderm (ec). (H) Internal focal view of the stage 9 embryo (1540–1620 h). (I) Surface view of the stage 10 embryo (1620–1720 h). (J) Internal focal view of stage 10 embryo, stomodaeum (sto). (K) Internal focal view of the early stage 11 embryo. (L) Stage 10 embryo treated with normal rabbit IgG as a primary antibody.

J). No significant staining signal was observed with embryos treated with the normal rabbit IgG as a primary antibody (Fig. 1L). At the end of the germ band extension, strong signals were associated with the neuroblast cells (Fig. 1K).

Maternal and Zygotic Expression of the PCNA *lacZ*

The regulatory region of *Drosophila* PCNA gene is composed of a promoter region (−168 to +24) and an upstream homeodomain protein binding region (−357 to −165) (Yamaguchi et al., 1991b). Transgenic flies carrying two different constructs of the plasmids p5′-607DPCNA-*lacZ*W8HS and p5′-168DPCNA-*lacZ*W8HS were established to examine the effect of a homeodomain protein binding region on the expression pattern of PCNA *lacZ*. The maternal and zygotic expressions of the PCNA *lacZ* were distinguished by comparing staining patterns in embryos from the cross of transgenic female × *w* male with that of *w* female × transgenic male.

In embryos from the cross of transgenic female × *w* male embryos, maternal storage of the PCNA *lacZ* in early embryos undergoing nuclear division cycle was detected (Fig. 2A,F). No significant difference was observed between flies carrying the p5′-607DPCNA-*lacZ*W8HS and those carrying the p5′-168DPCNA-*lacZ*W8HS. The PCNA *lacZ* protein appeared to be relatively stable, because the staining signal that appeared to come from maternal stores remained until the end of the germ band extension. Staining signals in Fig. 2 (panels E and J) are much stronger than those in Fig. 3 (panels J and T).

In embryos from transgenic male × *w* female, no detectable expression of the PCNA *lacZ* was observed at syncytial blastoderm and cellular blastoderm stages (Fig. 3A,B,K,L). Zygotic expression of the PCNA *lacZ* became detectable at the gastrulating stage, although the signal was weak (Fig. 3C,M). In the early phase of germ band extension, strong staining of the PCNA was seen in regions corresponding to the cephalic furrow, the amnioproctodeal invagination, the anterior midgut primordium, and the invaginated mesoderm (Fig. 3D–F,N–P). This expression pattern is similar to the distribution of endogenous PCNA protein, hence the PCNA *lacZ* fusion constructs seem to reproduce expression of the endogenous PCNA gene. At later stages, anti-*lacZ* staining was observed throughout the germ band and also in the cephalic region, including the anterior midgut pri-

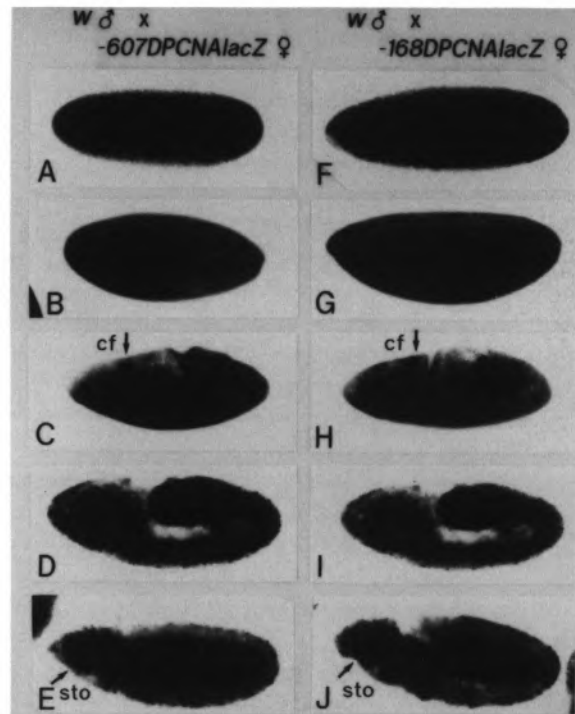


FIG. 2. Maternal contribution to PCNA *lacZ* expression. Embryos from *w* males × p5′-607DPCNA-*lacZ*W8HS females (A–E) or *w* males × p5′-168DPCNA-*lacZ*W8HS females (F–J), as indicated at the top, were immunostained with anti-*lacZ* antibody. (A,F) Surface views of syncytial blastoderm embryos (stage 4, 1320–1410 h). (B,G) Surface views of cellular blastoderm embryos (stage 5, 1410–1450 h). (C,H) Surface views of stage 7 embryos (1300–1510 h), cephalic furrow (cf). (D,I) Internal focal views of stage 8 embryos (1510–1540 h). (E,J) Internal focal views of stage 10 embryos (1620–1720 h), stomodeum (sto).

mordium, as well as in the posterior region, including the posterior midgut primordium and the proctodeal primordium (Fig. 3G–J,Q–T). No expression of PCNA *lacZ* was detected in the dorsal region, including the aminoserosa, throughout embryogenesis. No significant difference was observed between transgenic flies carrying the p5′-607DPCNA-*lacZ*W8HS and those carrying the p5′-168DPCNA-*lacZ*W8HS.

Because analyses by immunostaining are qualitative, quantitative measurements of β-galactosidase activity in the extracts of *Drosophila* bodies at various stages of development were made. Male flies from eight of 25 transformant lines carrying p5′-607DPCNA-*lacZ*W8HS and those from six out of 13 carrying p5′-168DPCNA-*lacZ*W8HS were crossed with wild-type females to observe the zygotic expression of the *lacZ*. Averaged values

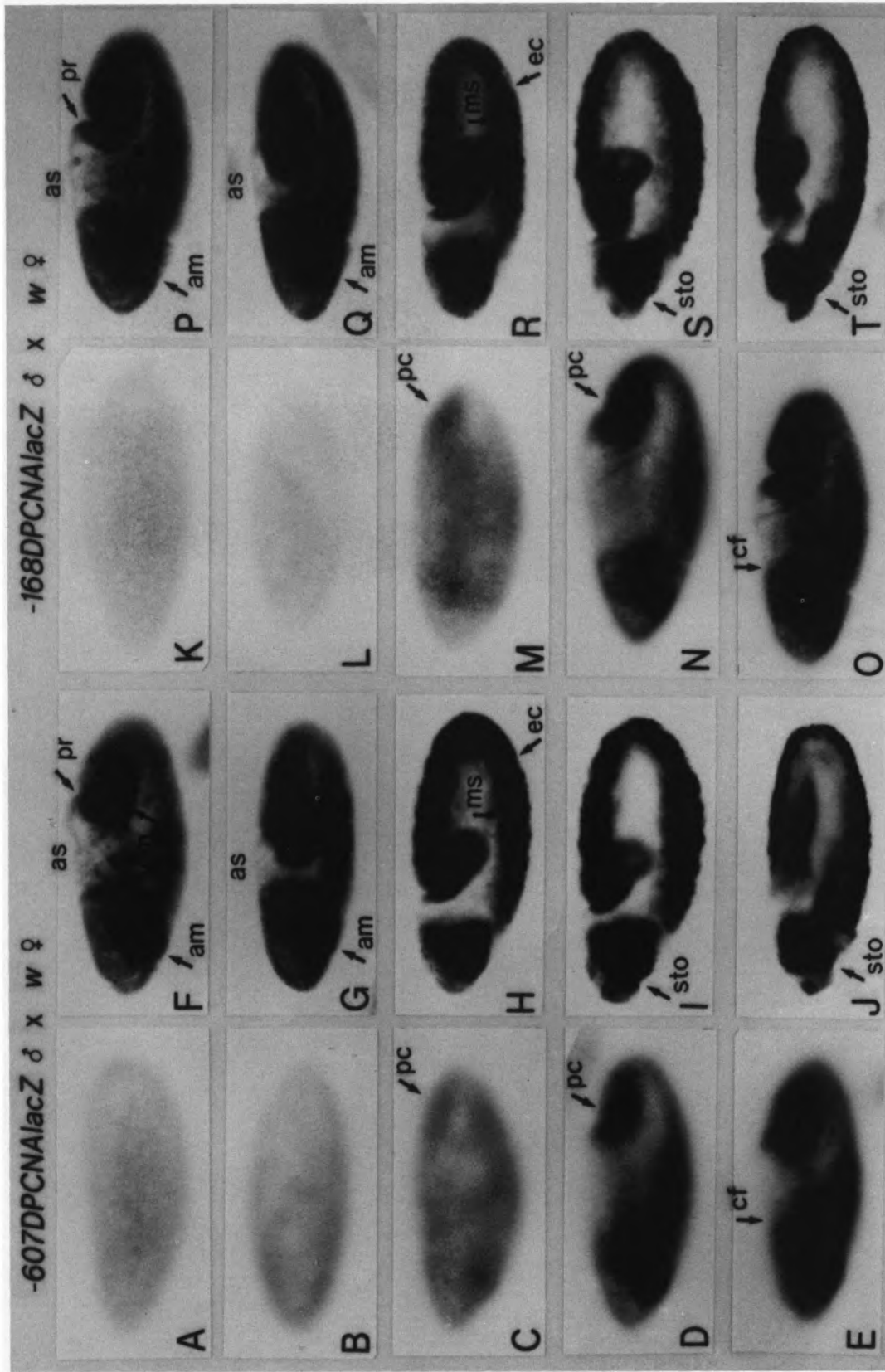


FIG. 3. Zygotic expression of PCNA *lacZ* in embryos. Embryos from p5'-607DPCNA/*lacZ*W8HS males × w females (A-J) or p5'-168DPCNA/*lacZ*W8HS males × w females, as indicated at the top, were immunostained with an anti-*lacZ* antibody. Surface views of (A,K) syncytial blastoderm embryos (stage 4, 1320–1410 h), (B,L) cellular blastoderm embryos (stage 5, 1410–1450 h), (C,M) early gastrulating embryos (stage 6, 1450–1500 h), (D,N) embryos at the beginning of germ band elongation (early stage 7, 1500–1510 h), and (E,O) embryos at the early phase of germ band elongation (late stage 7) are shown; pole cells (pc) and cephalic furrow (cf). (F,P) Internal focal views of embryos shown in (E,O), anterior midgut primordium (am), proctodeum primordium (pr), posterior midgut primordium (pm), and aminerosa (as). Internal focal views of (G,Q) stage 8 embryos (1510–1540 h), (H,R) stage 9 embryos (1540–1620 h), (I,S) stage 10 embryos (1620–1720 h), and (J,T) stage 11 embryos (1720–1920 h) are shown; mesoderm (ms), ectoderm (ec), stomodeum (sto).

for β -galactosidase activities at various developmental stages are shown in Fig. 4. The expression of *lacZ* was high in embryos, first and second instar larvae, and adult females and low in bodies of other stages of development. High expression of *lacZ* in the larva likely reflects the requirement of PCNA for the polytenization of larval tissues and cell proliferation in the imaginal discs. High expression of the *lacZ* in adult females represents the maternal contribution, as the expression was very low in adult males. Only a marginal difference was observed between transgenic flies carrying two different constructs of the transgene (Fig. 4). These results suggest that function of the homeodomain protein binding region located upstream of the PCNA gene is, if any, for the minor modulation of the promoter activity during normal developmental processes.

Expression Pattern of PCNA *lacZ* in Embryos Homozygous for *tor* Mutation

In ongoing work, we observed the ectopic expression of PCNA *lacZ* in embryos homozygous for *zen* mutation. The *zen*, a homeobox gene, belongs to dorso-ventral genes. Therefore, we conducted genetic crossing experiments of transgenic flies with several other homeobox gene mutant flies, including *Antp*, *Dfd*, *ftz*, *pb*, *Scr*, and *AbdB*. No significant change in the PCNA *lacZ* expression pattern was observed with these crossings (data not shown).

We then extended the analysis with the *tor* mutant flies. The *tor* gene is a maternal effect gene and plays a key role in the formation of terminal structures of the embryo (Nüsslein-Volhard et al., 1987). The *tor* gene product acts within the

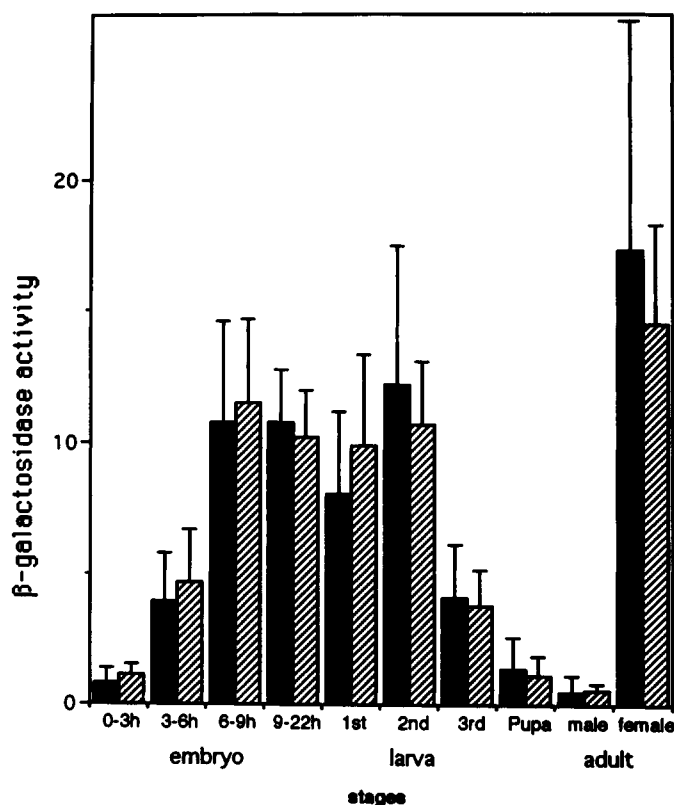


FIG. 4. Expression of PCNA *lacZ* during development. Male transgenic flies were crossed with female wild-type flies and extracts were prepared from *Drosophila* bodies at various stages of development. The β -galactosidase activities in the extracts are expressed as absorbance units per h per mg protein. Black bars indicate the averaged value of eight independent transgenic strains carrying the p5'-607DPCNA*lacZ*W8HS. Hatched bars indicate the averaged value of six independent strains carrying the p5'-168DPCNA*lacZ*W8HS. Deviation between independent strains is also indicated.

embryo to induce the expression of zygotic genes required for the development of terminal structures. Loss-of-function *tor* mutations result in truncation of the anterior head skeleton, and structures posterior to the seventh abdominal segment do not form (Shupbach and Wieschaus, 1986). As no corresponding invagination is formed at the posterior end of the embryo, the pole cells are often left outside the embryo. The germ band does not extend along the dorsal side of the embryo; rather, it forms in abnormal folds at the ventral side of the embryo (Weigel et al., 1990). In contrast, embryos derived from the *tor* gain-of-function mutant mothers exhibit a phenotype opposite that of embryos without *tor* activity (Klingler et al., 1988). The terminal parts of the anlagenplan are expanded at the expense of the central segmented region of the anlagenplan. The *tor*^{RL3} phenotype is caused by ectopic activation of the *tor* gene product at the central part of the embryos, which is normally restricted to the terminal regions (Casanova and Struhl, 1989). This is reflected by expression patterns of *ftz*, which is restricted to the center of the embryo (Klingler et al., 1988).

Male flies carrying the PCNA *lacZ* transgene were crossed with female flies homozygous for *tor* loss-of-function allele, *tor*^{PM}, or *tor* gain-of-function allele, *tor*^{RL3}. Although there was a slight repression of the *lacZ* expression at the posterior region of the *tor*^{PM} embryos (data not shown), much more extensive change of the *lacZ* expression was observed with the *tor*^{RL3} embryos. As shown in Fig. 5, PCNA *lacZ* expression in the cephalic region, as well as in the posterior end region, was observed in *tor*^{RL3} embryos carrying either the p5'-607DPCNA*lacZ*W8HS or the p5'-168DPCNA*lacZ*W8HS. The PCNA *lacZ* expression in the region throughout the germ band was also detected with embryos carrying the p5'-168DPCNA*lacZ*W8HS (Fig. 5E-H). In contrast, no expression in the central part of the presumptive mesodermal region was observed with embryos carrying the p5'-607DPCNA*lacZ*W8HS (Fig. 5A-D). In light of these results, the upstream homeodomain protein binding region of the PCNA gene is probably responsible for repression of the PCNA *lacZ* expression in the central region of the *tor*^{RL3} embryos.

The expression pattern of endogenous PCNA gene in the *tor*^{RL3} embryos was also examined by immunostaining with the anti-PCNA antibody. As shown in Fig. 1 (panels C-F) and Fig. 6 (panels A-C), strong staining with the anti-PCNA antibody was observed in the region surrounding the

ventral furrow of the wild-type embryo. In contrast, no or very little staining was observed in the central part of this region of the *tor*^{RL3} embryo (Fig. 6D-I). Therefore, *torso* is apparently involved in regulating both the transgene and the endogenous wild-type PCNA gene.

DISCUSSION

The binding consensus for homeodomain proteins that are located in the region from nucleotides -165 to -357 of the *Drosophila* PCNA gene is capable of binding homeodomain proteins in vitro (Yamaguchi et al., 1990). Interestingly, similar consensus sequences were found in the 5'-flanking regions of genes for the 180 kDa catalytic subunit (Hirose et al., 1991) and the 73 kDa subunit (Cotterill et al., 1992) of *Drosophila* DNA polymerase α . These observations suggest the possible involvement of homeodomain proteins in the regulation of *Drosophila* DNA replication-related genes. The present studies on transgenic flies revealed a minor role for the homeodomain protein binding region in the regulation of PCNA gene expression. Despite the minor role in the normal developmental process, the homeodomain protein binding region has the potential to regulate the PCNA gene expression extensively, because a significant difference in the *lacZ* expression pattern was observed between transgenic flies carrying the homeodomain protein binding region and those not carrying it, in the background of *tor* mutation.

Axton et al. (1994) have identified the *plutonium* (*plu*) gene that codes for the inhibitor of DNA replication; it locates in the 5'-flanking region of the PCNA gene. The *plu* gene is separated from the PCNA gene by less than 150 bp and the homeodomain protein binding region locates in the second intron and the 3' exon of the *plu*. Thus, the homeodomain protein binding region may play a major role in regulating expression of the *plu* gene.

The *tor* gene is a maternal effect gene and its product is a key component for the formation of embryo terminal structures (Schupbach and Wieschaus, 1986; Klingler et al., 1988; Sprenger et al., 1989; Casanova and Struhl, 1989). Activation of the *tor* protein leads to phosphorylation of subordinate regulatory molecules, an event that controls the activation of multiple genes responsible for generating terminal structures. Several downstream genes such as *Draf-1* (Nishida et al., 1988), *tailless* (Pignoni et al., 1990), *huckebein* (Weigel et al., 1990), and *forkhead* (Weigel et al., 1989)

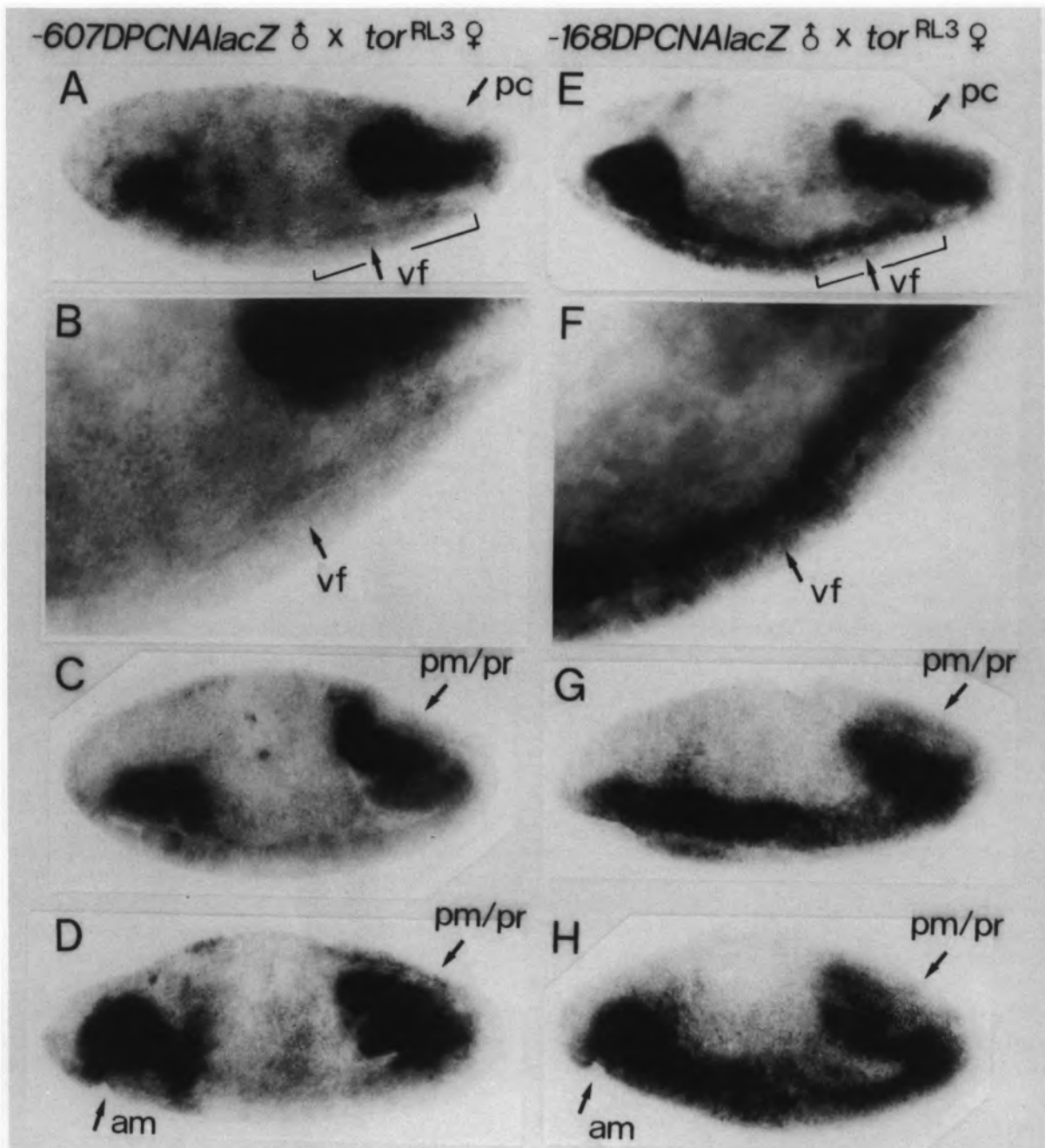


FIG. 5. Expression of PCNA *lacZ* in *tor*^{RL3} mutant embryos. Embryos from p5'-607DPCNA*lacZ*W8HS males × *tor*^{RL3} females (A-D) or p5'-168DPCNA*lacZ*W8HS males × *tor*^{RL3} females (E-H), as indicated at the top of each panel, were immunostained with anti-*lacZ* antibody. Surface views of 3-6-h embryos are shown. Stages of the embryos shown in (D) and (H) are more advanced than those shown in (A-C) and (E-G). In (B) and (F), indicated parts of (A) and (E) are shown in higher magnifications; ventral furrow (vf), posterior midgut primordium (pm), proctodeum primordium (pr), anterior midgut primordium (am).

have been identified in this signal transduction system. We attempted genetic crossing experiments of transgenic flies with some of these downstream mutants, but no significant change of expression pattern of the PCNA *lacZ* was obtained. Therefore, global change of expressions of multiple genes as a result of mutation in a master gene

like *torso* may be required to enhance the effect of the homeodomain protein binding region on PCNA promoter function. As none of the homeobox gene mutants so far examined affected the PCNA *lacZ* expression pattern, the involvement of multiple redundant genes in the regulation of the PCNA gene would need to be considered.

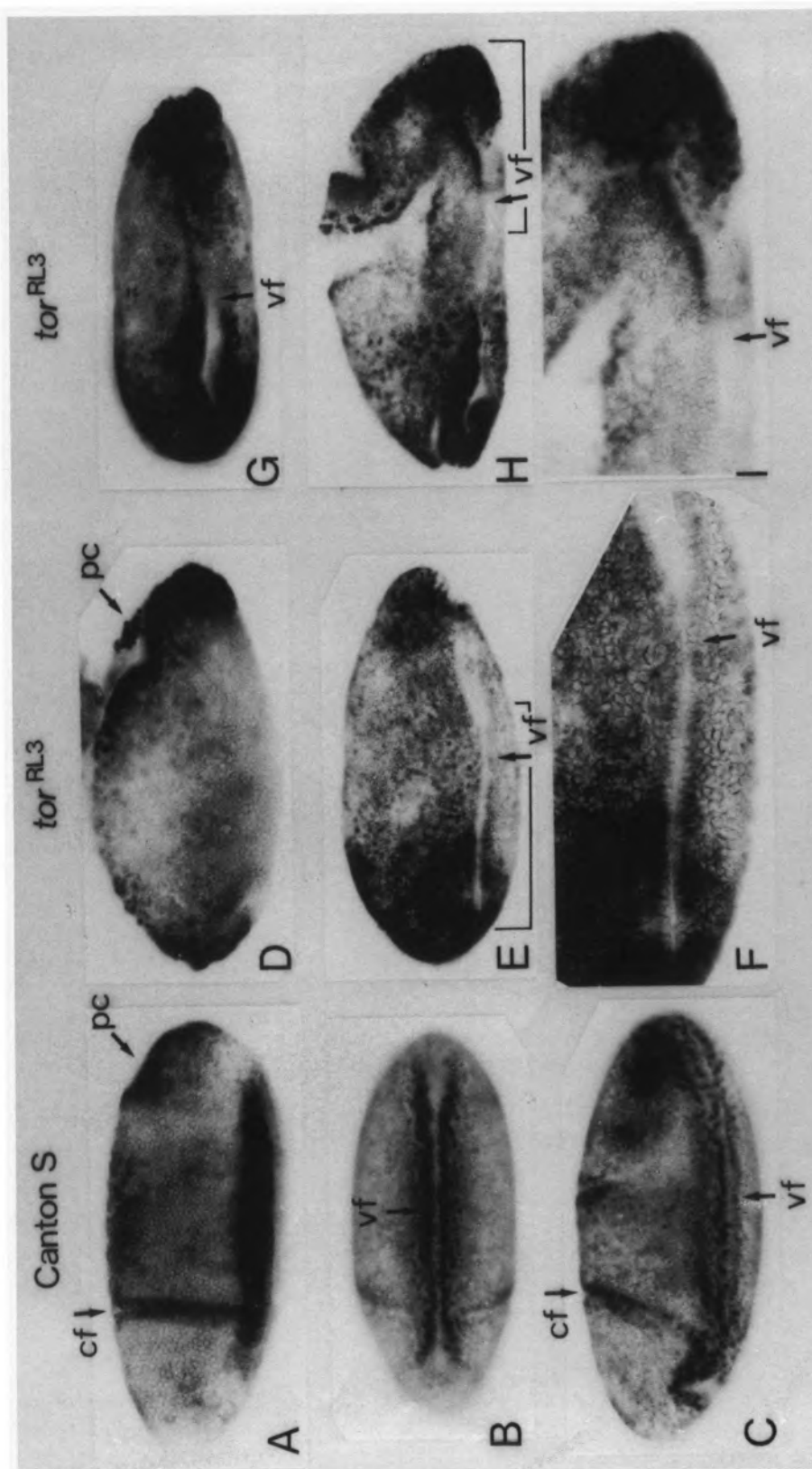


FIG. 6. Distribution of PCNA protein in tor^{RL3} mutant embryos. Whole-mount specimens of fixed embryos were immunostained with an anti-PCNA antibody. Embryos are oriented so that anterior aspects are to the left and ventral areas are down [except (B) and (G)]. (A) Surface view of the early gastrulating wild-type (Canton S) embryo (stage 6, 1420–1500 h). (B) Ventral surface view of the early gastrulating wild-type embryo (stage 6). (C) Surface view of the wild-type embryo at initiation of germ band elongation (stage 7, 1500–1510 h). (D–I) Surface views of 2–6-h tor^{RL3} embryos. Ventral surface view is shown in (G). Stage of the embryo shown in (H) is more advanced than those in (D–G). In (F) and (I), indicated parts of (E) and (H) are shown in higher magnifications; cephalic fold (cf), pole cell (pc), ventral furrow (vf).

In the *tor* gain-of-function mutant, *tor*^{RL3} embryo, expression of PCNA *lacZ* in the central region of the embryo was lost, thereby suggesting that *tor* appears to negatively regulate PCNA gene expression. Unknown homeodomain proteins may be involved in the downstream of *tor*. The structure of the central region is not formed in this mutant; therefore, accurate regulation of DNA replication-related genes like PCNA by the *tor*-mediated signal transduction system is vital for formation of a normal body.

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